DOCTOR OF MEDICINE

Calcium homeostasis and blood pressure regulation in normal and insulin-dependent diabetic pregnancy

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Award date:
1999

Awarding institution:
University of Bath

CALCIUM HOMEOSTASIS AND BLOOD PRESSURE REGULATION IN NORMAL AND INSULIN-DEPENDENT DIABETIC PREGNANCY

Submitted by
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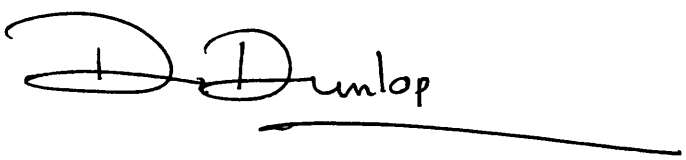
for the degree of M.D. of the University of Bath
1999



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ACKNOWLEDGEMENTS

Many people have helped me with these studies in both professional and volunteer capacities. In particular I would like to thank:

My supervisors Dr J P O'Hare, now consultant physician and diabetologist at The Hospital of St Cross, Rugby who provided initial ideas and support, and Professor F Broughton Pipkin, Professor of Perinatal Physiology at The University of Nottingham who gave invaluable advice and statistical knowledge at all times and who kept me going whenever the temptation to give up was too great!

Mr G Dunster, Consultant Obstetrician & Gynaecologist at The Royal United Hospital, Bath who allowed me to study his patients, taught me about the management of IDDM pregnancy and was always a source of encouragement.

Dr F Havard, in The Wolfson Centre, The Royal United Hospital, Bath who provided useful practical advice on the handling and storage of specimens.

Shire Pharmaceuticals for the calcium supplements and the Pharmacy Staff of Royal United Hospital, Bath who randomized the volunteers and dispensed the supplements.

Dr S. Woodhead and Dr Rhys John of the University Hospital, Wales who facilitated the intact parathyroid hormone measurements; Yvonne Cartwright, Senior Medical Laboratory Scientific Officer in the biochemistry laboratory of the Royal United Hospital, Bath who arranged the biochemical assays; and Suzanne Cooper and Averil Warren in Nottingham who performed the PRC and PRS assays.

All the volunteers who willingly gave up their time, blood and urine, and their friends and relatives who provided childcare whilst the volunteers were in the laboratory.

My husband and children who have tolerated the early hours when I left for the laboratory and the time spent away from them whilst I was engaged in "writing up".

My parents for support and encouragement throughout my career and for babysitting!

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACTH	Adreno-corticotrophic hormone
Ang I	Angiotensin I
Ang II	Angiotensin II
Ang III	Angiotensin III
ANOVA	Analysis of variance
Aogen	Angiotensinogen
AT	Angiotensin
ATP	Adenosine triphosphate
BP	Blood pressure
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
COC	Combined oral contraceptive pill
CT	Calcitonin
CV	Coefficient of variation
DBP	Diastolic blood pressure
DCT	Distal convoluted tubule
DHCC	Dihydroxycholecalciferol
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
FE	Fractional excretion
FRC	Follicular renin concentration
FRS	Follicular renin substrate
HCC	Hydroxycholecalciferol
hCG	Human chorionic gonadotrophin
HMW	High molecular weight
iCa^{2+}	Ionized calcium
IDDM	Insulin dependent (type 1) diabetes
IGF	Insulin-like growth factor
IOL	Induction of labour
iPTH	Intact parathyroid hormone
IQR	Interquartile range
JG	Juxta-glomerular

K	Potassium
LSCS	Lower segment Caesarean section
Mg	Magnesium
Na	Sodium
NICU	Neonatal intensive care unit
NIDDM	Non-insulin dependent (type 2) diabetes mellitus
PCT	Proximal convoluted tubule
PE	Pre-eclampsia
PG	Prostaglandin
pH	Negative log10[hydrogen ion concentration in mmol/L]
PIH	Pregnancy induced hypertension
PL	Placental lactogen
PO_4	Phosphate
PRA	Plasma renin activity
PRC	Plasma renin concentration
PRS	Plasma renin substrate
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
RAS	Renin-angiotensin system
SBP	Systolic blood pressure
SD	Standard deviation
SEM	Standard error of the mean
UCE	Urinary calcium excretion
UE..PM	Urinary excretion of .. per minute
UKPDS	United Kingdom Prospective Diabetes Study

ABSTRACT

Despite advances in care for individuals with insulin-dependent diabetes mellitus (IDDM) over the last seventy five years the management of diabetic pregnancy still presents a challenge to the medical profession with perinatal morbidity and mortality, and maternal morbidity remaining higher than in normal pregnancy. One cause of this is pre-eclampsia which is twice as common in IDDM pregnancy.

Blood pressure control is multi-factorial. Among systems involved are the calcium/phosphate/magnesium homeostatic mechanisms and the renin/angiotensin/aldosterone system. These are disordered in non-pregnant IDDM but have only been studied minimally in IDDM pregnancy. This thesis describes studies performed on normal and IDDM women during and after pregnancy:

- Assessment of dietary calcium
- A prospective, randomized, double-blind, placebo-controlled trial on the effect on blood pressure of a daily 1.5g calcium supplement, given from 20 weeks until delivery
- A longitudinal study of the short term effects of a standard meal and 1g oral calcium load on calcium homeostasis and plasma renin and substrate concentrations

Results presented show that both groups of women received adequate dietary calcium during pregnancy and that calcium supplementation did not affect blood pressure. Pregnancy-induced changes in blood pressure are different in IDDM women who have no mid-trimester fall. In IDDM, compared with normal pregnancy serum ionized calcium and urinary calcium excretion are identical, serum magnesium does not fall and intact parathyroid hormone concentrations are lower. Phosphate excretion is increased in pregnant and non-pregnant IDDM women.

Plasma renin concentration correlates negatively with serum ionized calcium in normal but not IDDM women; during pregnancy plasma renin concentration rises in normal but not IDDM women . Plasma renin substrate rises throughout normal

pregnancy, in IDDM pregnancy there is no further rise beyond 20 weeks. These observations suggest impaired synthesis or release of renin in IDDM with additional inhibitory factors acting in IDDM pregnancy.

The observed differences between normal and IDDM pregnancy, whilst interesting in their own right, do not suggest a mechanism for the increased incidence of PE in IDDM pregnancies.

INTRODUCTION

This thesis will investigate the hypothesis that pregnant insulin-dependent diabetic (IDDM) women have abnormalities in both calcium metabolism and the renin-angiotensin system (RAS) which predispose them to the development of pre-eclampsia (PE). It will also look in detail at the effect of calcium supplementation on these systems and on arterial blood pressure in normal and IDDM pregnancy. The remainder of this chapter provides an overview introduction to these various components, linking them where the current state of knowledge allows.

1.1 Historical perspective

Before the introduction of insulin in 1922 the only treatment for diabetes was "planned undernutrition", a system of dietary manipulation which verged on starvation. If young women survived they tended to be amenorrhoeic and therefore infertile.

One of the first reported cases of diabetic pregnancy described a woman who developed intense thirst and polyuria in her fourth pregnancy and subsequently delivered a stillborn infant weighing 12 pounds (Bennewitz, 1824). In 22 cases of diabetic pregnancy, presented to the London Obstetrical Society in 1882, perinatal mortality was 43%, 4 women died in coma at the time of delivery and 7 more died within the next 2 years. American figures were similar with maternal mortality for diabetic women in the Joslin Clinic between 1889 and 1917 being 34% and perinatal loss 60% (Hare & White, 1977).

With the introduction of insulin more diabetic women survived and became pregnant. Maternal mortality fell to 9.3% but fetal loss remained high at 45.2% (Skipper, 1933). Throughout the twentieth century regimes of tight maternal glucose control and improved paediatric care, including early feeding, for the infant have continued to improve these figures. In 545 diabetic pregnancies at King's College Hospital, London between 1971 and 1986 there was only one maternal death, in a woman with advanced diabetic nephropathy, and the perinatal mortality rate was 2.8% (Brudenell, 1989). More recently the Northern Diabetic Pregnancy Audit reported a perinatal mortality

rate of 38/1000 for diabetic pregnancy compared to the background rate of 9/1000 (Hawthorne, Robson, Ryall, Sen, Roberts & Ward-Platt, 1996). As this illustrates all the problems attending diabetic pregnancy have not been solved and they continue to have an increased complication rate when compared with non-diabetic pregnancies.

The measurement of arterial blood pressure is an integral part of the examination of every pregnant woman mainly because it is used both to detect and define PE and eclampsia. These pregnancy-specific disorders remain common, poorly understood, serious and sometimes fatal. Between 1994 and 1996 PE and its sequelae were responsible for 20 direct maternal deaths, a rate of 9.1 per 1 000 000 maternities, making it the second commonest cause of maternal mortality in the United Kingdom after thromboembolism (21.8 per 1 000 000); the overal direct maternal mortality rate for the same period was 74 per 1 000 000 (HMSO, 1998).

The incidence of PE is difficult to determine because of a lack of uniformity of definition between authors (see below). It is, however, generally accepted to be approximately twice as common in diabetic pregnancy as in non-diabetic pregnancy (Brohman, 1983; Garner, D'Alton, Dudley, Huard & Hardie, 1990) but the reason for this as well as the aetiology of PE remains unknown. Angiotensin II (Ang II) is one of the most potent vasoconstrictors known to man and it is now well established that sensitivity to this substance is reduced in normal pregnancy (see below). In non-diabetic women abnormalities in the RAS have been demonstrated in hypertensive pregnancy (Symonds & Broughton Pipkin, 1978) and subsequently Broughton Pipkin showed that in diabetic pregnancy the plasma renin and aldosterone concentrations are higher than in non-diabetic pregnancy and the plasma renin substrate (PRS) is lower (Broughton Pipkin, Hunter, Oats & O'Brien, 1982). It is possible that abnormalities within the RAS system of diabetic subjects may increase the frequency of pregnancy-induced hypertension and PE.

For many years it has been known that the renal excretion of calcium is reduced in PE. Recently much interest has been focused on calcium supplementation in the prevention of pre-eclampsia (Belizan, Villar, Zalazar, Rojas, Chan & Bryce, 1983[a]; Belizan, Villar, Gonzalez, Campodonico & Bergei, 1991; Lopez-Jaramillo, Narvaez, Weigel & Yepez,

1989; Villar, Repke, Belizan & Pareja, 1987). Non-pregnant diabetic individuals have been shown to have multiple abnormalities in calcium metabolism - low levels of serum ionized calcium (iCa^{2+}) have been reported (Fogh-Anderson, McNair, Moller-Petersen & Madsbad, 1982 & 1983), intact parathyroid hormone (iPTH) is low in diabetic subjects (Thalassinos, Hadjiyanni, Tzanela, Alevizaki & Philokiprou, 1993), urinary calcium excretion is increased (McNair, Madsbad, Christensen, Christiansen, Faber, Binder & Transbøl, 1979) and there is reduced bone mineralization in insulin-dependent diabetes (McNair, Madsbad, Christiansen, Faber, Transbøl & Binder, 1978[a]).

Four years after work on this thesis began the results of the National Institutes of Health trial of calcium supplementation to prevent PE have been published and show no beneficial effect of calcium supplements in normal healthy nulliparous women whose diet already contains adequate calcium, in preventing either PE or pregnancy-induced hypertension (PIH) (Levine, Hauth, Curet, Sibai, Catalano, Morris, DerSimonian, Esterlitz, Raymond, Bild, Clemens & Cutler, 1997). However IDDM women were excluded from this trial and for the reasons, alluded to above, and dealt with more fully later, their response to calcium supplementation may well differ from the normal response. To the author's knowledge this has not been investigated before.

1.2 CALCIUM HOMEOSTASIS

Calcium is the fifth most abundant element in the human body with an average 70kg adult containing 1100g of calcium. Calcium plays essential roles in muscle contraction, neural transmission, blood coagulation, membrane transport and enzyme activity, while as calcium phosphate it provides skeletal strength (Guyton, 1986).

Calcium metabolism consists of several intricate and interrelated mechanisms which normally operate to maintain the concentration of iCa^{2+} in the extracellular fluid (ECF) within narrow physiological limits. The metabolism of other minerals such as Mg (Mg) and inorganic phosphate (PO_4) are linked with calcium metabolism. Like calcium Mg is a divalent cation among the transition elements and so it is almost impossible to think of the physiology of one in isolation from the other. Hormonal control is exerted by parathyroid hormone (PTH), calcitonin (CT) and the vitamin D group of steroid hormones.

Serum calcium (normal range 2.25 - 2.65 mmol/l) is present in three different forms: approximately 40% is bound to plasma proteins, especially albumin; about 9% forms complexes with citrates and phosphates in such a way that it is not ionized and the remainder is ionized Ca^{2+} and can diffuse through the capillary membrane (Guyton, 1986). Anything which causes the concentration of albumin to rise or fall will have a parallel effect on the total serum calcium but the iCa^{2+} remains unchanged, controlled by the mechanisms described below.

1.2A Calcium absorption

In the non-pregnant state the recommended minimum adult daily intake of calcium is 900mg and in pregnancy 1250mg (Committee On Medical Aspects of food policy, 1991). The majority of this comes from dairy products as the calcium content of the non-dairy part of an average diet rarely exceeds 500mg. A pint of milk contains 700mg of calcium. Of note, this is in the whey not the curds, and so a low-fat diet containing skimmed or semi-skimmed milk contains marginally more calcium per pint. Almost 40% (350mg) of this dietary calcium is absorbed in the intestine but, as about

190mg daily is lost via intestinal secretions, overall only about one sixth of the total daily intake is utilized (Guyton, 1986).

Dietary absorption of calcium occurs in the small intestine by active transport and facilitated diffusion, both regulated by the 1,25-dihydroxycholecalciferol (DHCC) component of vitamin D – see below. The active transport mechanism is saturable whilst the rate of diffusion of calcium from the lumen of the intestine into the mucosal cells depends directly on the calcium concentration in the gut lumen, in other words the dietary calcium intake. In conditions of relatively high calcium intake and in areas of the intestine where the intra-luminal concentration of calcium is high the passive diffusion process predominates. However when calcium intake is low and the intra-luminal calcium concentration is less than 6mmol/L the active transport process is most important (Bringhurst & Potts, 1979). The same authors, on reviewing the evidence, suggest that "the greatest proportion of intestinal calcium absorption is accomplished in the ileum, especially under natural conditions where calcium is ingested along with solid food. The active transport process seems to predominate in the duodenum and jejunum and [predominates]....when the calcium content of the diet is low".

In addition to vitamin D physiological control of intestinal absorption of calcium is influenced by PTH but this is an indirect effect mediated by increased synthesis of 1,25-DHCC as discussed below.

1.2B Vitamin D

Several vitamin D compounds exist and are obtained either from the diet or by the action of ultraviolet on 7-dehydrocholesterol in the skin. The latter reaction produces vitamin D3 or cholecalciferol which is hydroxylated in the liver to 25-hydroxycholecalciferol (HCC) and this is then hydroxylated again, at the 1 position, in the kidneys to the biologically active 1,25-DHCC. Alternatively the 25-HCC can be hydroxylated at the 24 position to 24,25-DHCC. It appears that in vitamin D deficient states the major conversion is to the active 1,25-DHCC (Boyle, Gray & deLuca, 1971) whereas when the diet contains high levels of calcium and a source of vitamin D then the predominant conversion is to 24,25-DHCC (Holick, Schnoes, deLuca, Gray, Boyle

& Suda, 1972). The latter compound is rapidly metabolized in the liver and excreted in bile and so is unlikely to have a significant physiological role (Holick, Baxter & Schraufrogel, 1976).

The second hydroxylation from 25-HCC to 1,25-DHCC is the rate limiting step and so renal-1-hydroxylase activity, is the major determinant of intestinal calcium absorption in the non-pregnant state. Renal-1-hydroxylase is stimulated by PTH (Garabedian, Holick, deLuca & Boyle, 1972; Henry, 1979); low calcium (probably via PTH) and directly by low phosphate levels (Baxter & deLuca, 1976); various peptide hormones including growth hormone and prolactin (Spanos, Brown, Stevenson & MacIntyre, 1981); and oestrogen (Kenney, 1976; Castillo, Tanaka, deLuca & Sunde, 1977). The stimulatory effect of PTH on renal-1-hydroxylase is impaired in diabetes and this impairment is corrected by insulin replacement (Wongsurawat & Armbrecht, 1985). Conversely renal-1-hydroxylase is inhibited by negative feedback by 1,25-DHCC and low levels of PTH.

1.2C Parathyroid hormone

PTH is a small protein with a molecular weight of approximately 9 500, made up of 84 amino acids, secreted by the chief cells of the parathyroid glands. The human form was first isolated in small quantities by O'Riordan and colleagues in 1971. Subsequent work which extracted larger quantities of a more highly purified form of the hormone allowed its amino acid sequence to be established (Keutmann, Barling, Hendy, Segre, Niall, Aurbach, Potts & O'Riordan, 1974). Smaller compounds with some PTH activity have also been isolated from the parathyroid glands but this activity is always less than that of intact PTH and it seems likely that these compounds are breakdown products of PTH (Segre, 1979).

PTH secretion is regulated predominantly by the ionized fraction of serum calcium being increased by hypocalcaemia and suppressed by hypercalcaemia but the relationship is not linear; instead it is sigmoid so that changes in PTH secretion occur over a narrow range of serum iCa^{2+} which corresponds with the lower end of the physiological range (fig. 1.2i). Thus if serum iCa^{2+} is falling and serious hypocalcaemia

is threatened then scretion of PTH is maximal and so serum iCa^{2+} is maintained, whereas if serum iCa^{2+} is well within the physiological range PTH secretion is minimal.

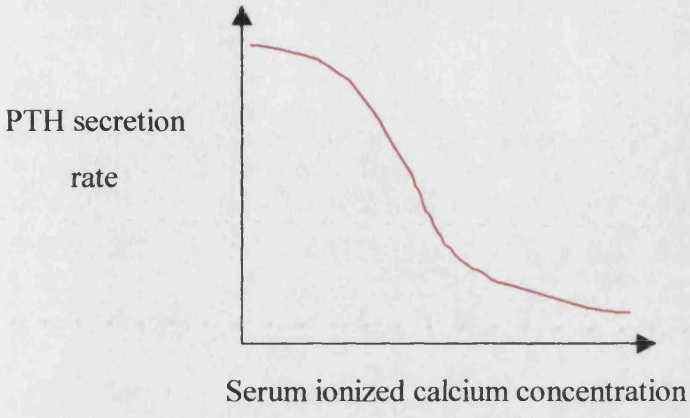


Fig.1.2i The relationship between serum iCa^{2+} and PTH secretion (after several authors)

Additional factors also regulate PTH secretion. As mentioned above PTH influences vitamin D metabolism and it appears that there is a reciprocal relationship with metabolites of vitamin D such as 24,25-DHCC, which are produced in conditions of calcium plenty, having an inhibitory effect on PTH secretion (Bates, Care, Peacock, Mawer, & Taylor, 1975). Mg deficiency also impairs PTH release (Anast, Winnacker, Forte & Burns, 1976). Secretion of PTH is increased by adrenergic agonists via β receptors (Kukreja, Hargis, Bowser, Henderson, Fisherman & Williams, 1975) and by intravenous infusion of saline (Zemel, Bedford, Standley & Sowers, 1989). Plasma inorganic phosphate does not have a *direct* effect on PTH secretion although it *indirectly* increases plasma concentration of PTH by causing a fall in iCa^{2+} concentration.

1.2D Calcium excretion

As early as 1946 Elizabeth Knapp showed that in over 600 people studied, from age 1 to 80, urinary calcium excretion (UCE) increased with increasing calcium intake per unit weight. She also postulated that UCE was under the influence of an, as yet, unrecognised endocrine factor. Subsequently this factor was identified as PTH.

At normal glomerular filtration rates about 215 mmol of calcium is filtered daily but the average daily UCE is around 4mmol; this corresponds to 0.16g (Lemann, Adams & Gray, 1979). From this it can be seen that virtually all of the calcium filtered by the kidney is reabsorbed. This reabsorption is distributed throughout the kidney with approximately 60% occurring in the proximal tubule, 20 -25% in the Loop of Henle and the remainder in the distal tubule and collecting ducts (Bringhurst & Potts, 1979). No calcium is secreted by the kidney tubules.

In the proximal tubule calcium is reabsorbed actively and this process is closely linked to sodium reabsorption in dogs (Beck & Goldberg, 1973). In humans acute administration of sodium chloride has much less effect on UCE (Lemann *et al*, 1979). The active transport mechanism is voltage dependent but is not affected by PTH. The mechanism for calcium reabsorption in the Loop of Henle is controversial.

It is in the distal tubule and collecting ducts that calcium reabsorption is finely controlled depending on the calcium concentration of the blood. At low serum concentrations virtually no calcium is excreted but if the serum concentration rises above the normal range then urinary calcium excretion (UCE) increases markedly. Distal reabsorption of calcium is increased by raised levels of PTH which acts via cyclic adenosine monophosphate (cAMP); PTH also increases renal excretion of phosphate (Agus, Gardner & Beck, 1973).

The normal range of UCE is wide (Knapp, 1946 and Lemann *et al*, 1979) and it is difficult to set an upper limit of normal. Early studies suggested that it was mainly dependent on skeletal weight and dietary calcium intake (Knapp, 1946; MacFadyen, Nordin, Smith, Wayne & Rae, 1965; Peacock, Hodgkinson & Nordin, 1967). Subsequently a non-hospital based study on factors influencing UCE concluded that it "is determined more by the absorption of calcium from the intestine than by the intake of calcium, and that the efficiency of absorption decreases in men and women as they grow older" (Davis, Morgan & Rivlin, 1970).

UCE rises with ingestion or infusion of glucose (Lindeman, Adler, Yiexgst & Beard, 1967; Lemann, Lennon, Piering, Prien & Riccannati, 1970). In the study by Lemann

and colleagues serum ultrafiltrable calcium and Mg were measured and were shown not to be affected by an oral glucose load. The subjects then stood, a manoeuvre which had previously been shown to decrease inulin clearance and therefore the GFR. Despite this reduction in GFR the urinary excretion of both calcium and Mg rose suggesting that glucose is affecting the net tubular reabsorption of these cations. PTH was not thought to be likely to be responsible for this change in tubular function partly because the effect was too rapid and also because a similar effect was observed in individuals known to have deficient PTH secretion. Lemann concluded that "the inhibition of net reabsorption of calcium and Mg after glucose appears more likely to be a consequence of an alteration by glucose ..... of renal tubular cell metabolism".

Increased protein intake increases the UCE (Lindemann *et al,* 1967; Kerstetter & Allen, 1990) as does a metabolic acidosis produced by administration of ammonium chloride, (Lemann *et al*, 1979). The latter is probably the mechanism by which a high protein intake increases the UCE. Alcohol also increases UCE (Lindemann *et al,* 1967). Numerous studies by Lemann and colleagues over twenty years looked at the effect of dietary phosphate on UCE and concluded that except in cases of extreme phosphate deprivation, when UCE rose, it had little effect (Lemann *et al*, 1979).

1.2E Bone and its relationship to extracellular calcium

This aspect of calcium metabolism was not studied in detail in this thesis. Therefore a brief summary only of this important topic is given below.

The majority of the calcium present in the body is found in bone where it combines with phosphate to produce crystalline salts known as hydroxyapatites:

$$Ca^{2+}{}_{10-x}(H_3O^+)_{2x} \bullet (PO4)_6(OH^-)_2$$

The relative ratio of calcium to phosphorus in these long, flat crystals varies markedly under different nutritional conditions.

If the iCa^{2+} concentration of the blood is raised or lowered acutely it rapidly returns to normal. This is achieved by there being exchangeable calcium which is always in

equilibrium with the serum calcium. The majority of this exchangeable calcium is found in bone and accounts for 0.4 - 1% of the total bone calcium. This buffering effect of bone on serum calcium acts almost instantaneously and is not dependent on any hormones. Hormonal activity comes into play within a few minutes of a change in serum iCa^{2+} concentration. PTH increases serum calcium by promoting the resorption of bone, while the reverse activity is controlled by CT. CT acts mainly in the short term and is eventually over-ridden by the PTH control mechanism. Over a prolonged period of time it is the PTH system that sets the long-term level of iCa^{2+} in the ECF. As the buffering capacity of bone on the serum iCa^{2+} is vast PTH mainly acts via bone. However if the skeleton becomes saturated with calcium then the slight rise in serum iCa^{2+} which occurs leads to a fall in the secretion of PTH and so the intestinal and renal absorption of calcium is reduced and therefore serum calcium returns to normal (Guyton, 1986).

1.2F Calcium metabolism in pregnancy

Pregnancy creates great demands on maternal calcium stores; the mineral content of an average mature baby is about 28g (700mmol) of calcium and 16g of phosphorus, with 99% and 85% respectively in the skeleton. The combination of progressive skeletal mineralization and exponential growth means that some 70% of this calcium is deposited in the third trimester; the net flux of calcium into the fetus towards the end of pregnancy is about 250mg (6.25mmol) per day (Misra & Anderson, 1990). The control of this process is achieved by integration and collaboration between the fetal and maternal endocrine systems.

Maternal calcium physiology

Total calcium levels in the maternal serum decline during pregnancy paralleling a fall in albumin concentration produced by an increase in plasma volume (Pitkin, Reynolds, Williams & Hargis, 1979). This rise in plasma volume is approximately 1 250ml in healthy first pregnancies and more in subsequent pregnancies and mainly takes place before 32 -34 weeks gestation (Letsky, 1987). There is no consensus as to what happens to serum iCa^{2+} during pregnancy. Some report it as falling slightly (Tan,

Reman & Sinnathyray, 1972; Pitkin & Gebhardt, 1977; Pitkin *et al*, 1979), others have found that it rises (Reitz, Daane, Woods & Weinstein, 1977) and further studies have found no change (Gertner, Coustan, Kliger, Mallette, Ravin & Broadus,1986; Roelofsen, Berkel, Uttendorfsky & Slegers, 1988; Seely, Brown, DeMaggio, Weldon & Graves, 1997). Another group reported a more complex picture with an overall rise in pregnancy compared with 12 months post-partum but a slight fall in the latter part of the third trimester (Rasmussen, Frolich, Hornes & Hegedus, 1990). These discrepancies cannot simply be explained by variation in analysis technique, for example Tan *et al* (1972), Pitkin *et al* (1979) and Reitz *et al* (1977) all used the same calcium flow-through electrode. Only three of these studies (Pitkin *et al*, 1979; Seely *et al*,1997; Rasmussen *et al*, 1990) used the same women throughout the study so that each woman gave repeated blood samples and acted as her own non-pregnant control; the others used groups of different women at different gestations and this may partly account for the discrepancy in their results.

Although the longitudinal studies are likely to give a more accurate picture of pregnancy-induced changes in serum iCa^{2+} the post-partum data in the studies of both Pitkin *et al* (1979) and Seely *et al* (1997), are confused by the fact that some of the women were still lactating (20 of 30 women and 8 of 23 women respectively) when the blood samples were drawn. Pitkin compared iCa^{2+} levels in the lactating and non-lactating women and found no difference between the groups but this work has not been repeated with larger numbers (Pitkin *et al*, 1979). The study by Rasmussen *et al* (1990) did not mention whether or not the women were lactating when they gave blood samples.

Despite the increased demand for calcium in pregnancy the UCE rises markedly during normal pregnancy (Howarth, Morgan & Payne 1977; Gertner, Coustan, Kliger, Mallette, Ravin & Broadus, 1986; Roelofsen, Berkel, Uttendorfsky & Slegers, 1988; Macintosh, Hutchesson & Duncan, 1990; Seely *et al*,1997). A highly significant correlation between calcium excretion and creatinine clearance has been shown in pregnant women and it is likely that this hypercalciuria of pregnancy is caused by the increase in glomerular filtration rate which occurs during pregnancy (Howarth *et al*, 1977).

The increased renal loss of calcium in pregnancy may be partly compensated for by an increased oral intake of calcium but more significantly the proportion of dietary calcium which is absorbed rises in pregnancy (Heaney & Skillman, 1971; Gertner *et al*, 1986). Levels of 1,25-DHCC rise during pregnancy (Kumar, Cohen, Silva & Epstein, 1979; Whitehead, Lane, Young, Campbell, Abeyasekera, Hillyard, MacIntyre, Phang & Stevenson, 1981; Reddy, Norman, Willis, Goltzman, Guyda, Solomon, Philips, Bishop & Mayer, 1983; Gertner *et al,* 1986; Verhaeghe & Bouillon, 1992; Ardawi, Nasrat & A'Aqueel, 1997; Seely *et al,* 1997) and these higher levels mediate an increase in intestinal absorption of calcium. Thus it can be seen that pregnant women utilize available calcium more efficiently then non-pregnant women. This explains why repeated pregnancies are usually not associated with osteopenia (Walker, Richardson & Walker, 1971) although this is not the case for Asian immigrants to the West who are relatively vitamin D deficient because, for cultural reasons, they expose their skin less to sunlight than their Caucasian neighbours. Marya, Rathee & Manrow (1987) studied a group of 50 Hindu women, half of whom received vitamin D supplements. They found that the unsupplemented women were actually hypocalciuric compared with non-pregnant, non-lactating Hindu women controls, while those who received 600,000iu vitamin D showed the hypercalciuria of pregnancy previously demonstrated in Caucasian women.

The rise in circulating levels of 1,25-DHCC does not seem to be related to PTH concentration. Data on PTH concentrations in pregnancy are contradictory with some reporting an increase (Cushard, Creditor, Canterbury & Reiss, 1972; Pitkin *et al*, 1979), others a decrease (Reddy *et al,* 1983; Davis, Hawkins, Rubin, Posillico, Brown & Schiff, 1988) and some no change (Gillette, Insogna, Lewis & Baran, 1982). Much of the conflicting data on PTH concentration during pregnancy can be explained by discrepancies between the different assays used by different research groups. Immunoreactive assays measure not only the intact, and presumably bioactive, PTH molecule but also smaller, non-bioactive fragments of the complete hormone. Therefore if the ratio of intact PTH (iPTH) to breakdown fragments of PTH differs between the pregnant and non-pregnant states discrepant results will be obtained. Newer assays which measure iPTH have shown either no change in pregnancy (Mimouni, Tsang, Hertzberg, Neumann & Ellis, 1989; Saggese, Baroncelli, Bertelloni

& Cipolloni, 1991) or lower values during pregnancy, compared to postpartum (Rasmussen *et al,* 1990; Ardawi *et al,* 1997). Despite agreeing that iPTH was lower overall during pregnancy the latter authors disagreed about changes in serum iPTH during the course of pregnancy with Rasmussen *et al* (1990) reporting a rise between the second and third trimesters and Ardawi *et al* (1997) reporting a fall. Both were prospective longitudinal studies but the latter looked at twice as many women (40 vs 20) and so may be more indicative of the true state of affairs.

Much of the additional vitamin D found in pregnancy is produced in the placenta. The ability of human decidual tissue to generate 1,25-DHCC and of human placenta to generate both 1,25-DHCC and 24,25-DHCC *in vitro* was first reported in 1979 (Weisman, Harell, Edelstein, David, Spirer & Golander). Subsequently (1981) Whitsett, Ho, Tsang, Norman & Adams showed that human placenta could also synthesize 1,25-DHCC *in vitro.* As well as directly producing vitamin D the placenta secretes a protein hormone, placental lactogen (PL), which increases *in vitro* renal 1-alpha-hydroxylase activity (Spanos et al, 1981). Additionally PL stimulates the production of insulin-like growth factor 1 (IGF-1) in the mother and the fetus and this also increases renal 1,25-DHCC production (Caverzasio, Montessuit & Bonjour, 1990). Oestrogen, as mentioned above (section 1.2B, p13) also stimulates renal 1-alpha-hydroxylase activity and is a further cause of the raised levels of vitamin D found in pregnancy.

Levels of CT in human pregnancy have generally been reported as being higher than non-pregnant values in both animal studies (Reynolds, Williams & Pitkin, 1981) and humans (Whitehead *et al,* 1981; Sgambata, Passariello, Buoninconti, Caserta & Paolisso, 1986; Ardawi *et al,* 1997). It has been speculated that elevated CT protects the maternal skeleton from excessive resorption of calcium but this has not been proven. As in the non-pregnant state the exact physiological role of CT is still speculative.

Placental transport of calcium

This was not directly examined in this thesis and is therefore only discussed briefly in this introductory chapter; it has been recently reviewed extensively by Kovacs &

Kronenberg (1997). In summary calcium is actively transported across the placenta and this process involves both calcium-binding proteins located in the cytotrophoblastic cells and a Ca^{2+}-ATPase dependent pump located in the membrane on the fetal side of the placenta. Maternal hormones do not seem to be involved in the placental transfer of calcium and current thinking is that it is chiefly regulated by parathyroid hormone-related protein (PTHrP). PTHrP was first identified as the cause of the hypercalcaemia of malignancy in 1987 (Moseley, Kubota, Diefenbach-Jagger, Wettenhall, Kemp, Suva, Rodda, Ebeling, Hudson, Zajac & Martin). PTHrP as its name suggests has a strong resemblance to PTH and homology at its amino-terminal end means that it has similar actions on kidney and bone. However the mid-portion of the molecule has the additional role of acting as the main regulator of placental transport of calcium (MacIssac, Heath, Rodda, Moseley, Care, Martin & Caple, 1991). PTHrP is produced in many sites throughout the developing fetus including the fetal parathyroids and the placenta (Abbas, Pickard, Illingworth, Storer, Purdie, Moniz, Dixit, Caple, Ebeling & Rodda, 1990); it does not cross the placenta. PTHrP does not seem to be involved in neonatal calcium homeostasis, instead iPTH is the dominant hormone, as described below, in maintaining normocalcaemia.

Fetal and neonatal calcium physiology

Umbilical cord blood has been shown to contain higher levels of total calcium, iCa^{2+}, Mg, PO_4 and albumin (Pitkin, Cruikshank, Schauberger, Reynolds, Williams & Hargis, 1980). The same study showed that fetal CT levels were higher than maternal levels confirming earlier work (Samaan, Anderson & Adam-Mayne, 1975) but the significance of this is unknown. Within 2 hours of delivery fetal iCa^2 has fallen significantly ($p<0.01$) and this continues to fall within the first day of life. The neonate responds, as an adult would, by producing increasing amounts of iPTH (Loughhead, Mimouni, Ross & Tsang, 1990). The role of vitamin D in fetal calcium metabolism is uncertain; various animal experiments seem to indicate that it is not necessary for fetal calcium homeostasis or skeletal formation but nevertheless it is known that the fetus can produce both 1,25-DHCC and also its receptor suggesting that vitamin D must have an, as yet undetermined, physiological role in fetal life (Kovacs & Kronenberg, 1997).

1.2G Calcium and hypertension

The role played by extracellular iCa^{2+} in blood pressure homeostasis and, especially, in the pathophysiology of hypertensive disease has been increasingly appreciated in the last decade or so, although Ringer first noted a requirement for iCa^{2+} in maintaining cardiac muscle contraction in 1883, and Addison showed that oral calcium supplements lowered blood pressure in 1924. Recent research has looked at many aspects of calcium's involvement in hypertension from its intracellular second messenger function through to epidemiological and dietary work linking calcium intake and level of blood pressure. Work has also been done to see if any of the calciotrophic hormones, such as PTH play a part in the aetiology of hypertension.

The cellular basis of vasoconstriction

Contraction of vascular smooth muscle is initiated by depolarization of the cell membrane by an action potential. Unlike the situation in striated muscle, where the depolarization is caused by the influx of sodium ions (Na^+) alone, in smooth muscle both Na^+ and iCa^{2+} contribute. These ions then diffuse throughout the cell and activate adenosine triphosphatase (ATPase) activity in the heads of the myosin filaments. The energy released by dephosphorylation of the ATP allows the myosin filaments to pull along the actin filaments thus causing overall shortening of the muscle ie. vasoconstriction. Vascular relaxation occurs as a calcium pump gradually shifts the iCa^{2+} from intrcellular to extracellular pools (Guyton, 1986).

Therefore it can be seen that extracellular iCa^{2+} is essential to initiate smooth muscle contraction and it could be surmised that calcium deficiency might be associated with hypotension but this is probably only true in extreme deprivation states. However *in vitro* experiments with vascular strip preparations have shown that the relationship of extracellular calcium to the magnitude of an induced contraction is non-linear; in fact it is parabolic (fig. 1.2ii). This reduction in vascular reactivity with increasing concentrations of extracellular calcium may be due to the membrane-stabilizing effect of iCa^{2+}. Acute infusion of calcium in humans has not been shown to have an effect on blood pressure but long term oral dietary supplementation with calcium has been shown to lower it (Belizan, Villar, Pineda, Gonzalez, Sainz, Garrera & Sibrian, 1983[b]).

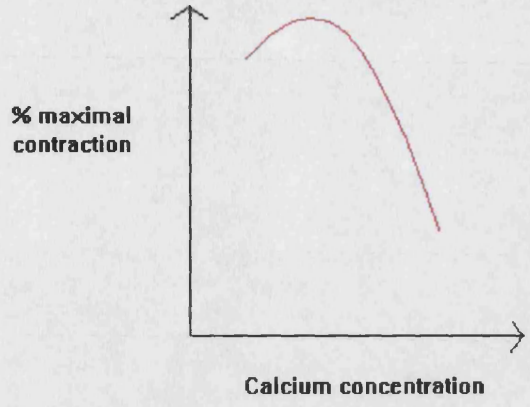


Figure 1.2ii The relationship between extracellular iCa^{2+} concentration and the change in vascular tissue tone

Dietary calcium and hypertension

On a larger scale, metabolic studies have looked at dietary intake, circulating levels and urinary excretion of calcium and have found many abnormalities in the hypertensive population. However the clinical data appears contradictory, probably reflecting the many subgroups of hypertension and their heterogeneous causes. Also most large studies assessed calcium intake only by dietary recall, which is an intrinsically inaccurate method, as it depends on individual memory. A further difficulty with studies involving thousands of individuals is that it is difficult to control for confounding factors.

Many studies suggest that a calcium deficient diet is associated with hypertension. Ackley, Barrett-Connor & Suare (1983) reported an inverse association between dietary calcium intake and blood pressure in a predominantly white Southern California community assessing dietary calcium both in terms of milk consumption and by total dairy product intake. Data from the American National Center for Health Statistics support this looking at a much larger population (10 372 individuals) of all races (McCarron, Morris, Henry & Stanton, 1984). They showed that calcium was "the nutrient for which reduced intake was the most consistent in hypertensive individuals". The inverse relationship held whether hypertension was defined as: systolic blood pressure (SBP) > 160, SBP >140 or the top 10% of the population.

Serum calcium and hypertension

Whilst the dietary association of reduced calcium intake and hypertension is strong there is less evidence to show a link between low serum levels of calcium, or iCa^{2+}, and hypertension. McCarron (1982) found lower fasting serum levels of iCa^{2+} in untreated mildly hypertensive subjects compared to normal controls; hypertension was defined as a diastolic blood pressure (DBP) >95mmHG or a mean arterial pressure >105mmHG. Strazzullo, Nunziata, Cirillo, Giannattasio, Ferrara, Mattioli & Mancini, (1982) found a non-significant reduction in both total serum calcium and serum iCa^{2+} in untreated essential hypertensive patients compared to controls and also a highly significant ($p<0.005$) increase in the UCE in the hypertensive group.

Whilst these studies link dietary *deficiency* of calcium and hypertension others have linked *excess* calcium to hypertension although not at a dietary level. In a large epidemiological study of 9 321 men Kesteloot & Geboers (1982) found a significant positive correlation between total serum calcium and both SBP and DBP.

This link between raised extracellular levels of calcium and high blood pressure seems to make more sense physiologically. Referring back to the parabolic relationship between extracellular iCa^{2+} concentration and the degree of vascular smooth muscle contraction it should be remembered that the physiological range of iCa^{2+} concentration is on the left hand side of the curve whilst supraphysiological levels(to the right hand side) are needed to produce relaxation. However it is likely to be high levels of *intracellular* iCa^{2+} which are important in causing increased vasoconstriction in hypertension while serum concentrations of iCa^{2+} are relatively unimportant; indeed they may even be reduced because more calcium is sequestered within cells than in normal individuals.

Spontaneously hypertensive rats, intracellular iCa^{2+} and hypertension

In vivo and *in vitro* a great deal of work has been done on animal models of human hypertension; in particular spontaneously hypertensive rats. This rat work has been summarized in a review article by McCarron (1985): in these hypertensive rats serum iCa^{2+} levels are low, PTH levels are raised and UCE is increased; acute administration

of calcium to spontaneously hypertensive rats has been shown to increase BP; at a cellular level isolated strips of femoral artery showed increased vasoconstriction when exposed to increasing concentrations of calcium in the perfusing solution compared with tissue from normotensive rats. Defects in the ability of red blood cell membranes to bind calcium have been shown in hypertensive rats and it appears that the activity of the membrane-bound Ca-ATPase pump is reduced which means that the ability of cells to buffer intracellular calcium is reduced. The permeability of vascular membranes to calcium is raised in hypertensive rats. This combination of increased membrane permeability and reduced intracellular buffering of calcium means that intracellular concentrations of calcium are raised in rats (McCarron, 1985). Human erythrocytes and platelets from hypertensive individuals have been compared to normotensive controls and have also been found to contain higher levels of iCa^{2+} (Robinson, 1984).

Overall therefore the data appears to be contradictory with excess calcium being associated with hypertension at a cellular level but decreased calcium intake on the whole body scale linked with raised blood pressure. This shows the drawback of crude dietary and population evidence which disregard the complexity of human calcium homeostasis which involves several hormones and organ systems as described. Nevertheless the dietary studies raise interesting questions which can only be resolved by much more detailed study of calcium homeostasis in man.

1.2H Hyperparathyroidism and hypertension

A link between hyperparathyroidism and hypertension was first reported by Hellstrom, Birke & Edvall in 1957. They defined hypertension as 150/100mmHg and found that 70% of patients with hyperparathyroidism were hypertensive at some stage of the disease; however they concluded that this was mainly due to a higher incidence of renal disease in the hyperparathyroid patients. However, Lafferty (1981) reported 100 consecutive cases of hyperparathyroidism of whom 42% were hypertensive, twice the prevalence found in an age and sex-matched control group; of the hyperparathyroid patients with hypertension, only 4 (10%) had raised serum creatinine levels.

In 1980 a possible mechanism for this was put forward by an American group who confirmed that serum PTH was higher in hypertensive individuals compared with controls and showed that for any given level of sodium excretion the hypertensive group had significantly higher calcium excretion levels. They hypothesized that " the increased [parathyroid] gland activity appears, in part, to be an appropriate, physiological response to a ..... renal calcium leak, associated with essential hypertension" (McCarron, Pingree, Rubin, Gaucher, Molitch & Krutzik, 1980). Subsequent work by Strazzullo *et al* (1983) confirmed that renal calcium excretion was increased in 55 hypertensive individuals compared with 55 age- and sex-matched controls and also showed significantly raised levels of iPTH which they felt was a secondary phenomenon caused by the renal wastage of calcium.

Work from Holland has also looked at iPTH and shown that this is increased in young (20-39 year old) primary hypertensive subjects. In this study hypertension was defined as ≥ 140/90mmHg but not sufficient to warrant treatment (Grobbee, Hackeng, Birkenhager & Hofman, 1988). The authors suggested that they might have identified a select group likely to develop significant hypertension in later years (analogous to the group of women who develop gestational hypertension) but in contrast to Strazzullo *et al* (1983) and Grobbee *et al* (1988) found no difference in urinary calcium excretion between their "hypertensive" and normotensive groups.

1.2J Calcium and hypertension in pregnancy

Following work on the normal and hypertensive populations interest has spread to the pregnant sub-population and the possible link between calcium and pregnancy-induced hypertension (PIH) or even pre-eclampsia (PE) and eclampsia.

In 1980 Belizan and Villar observed a very low incidence of pre-eclampsia and eclampsia in Guatemala where the overall nutrient intake is poor but the basic diet is high in calcium. Similar observations came from Ethiopia where people have a high calcium intake (derived from a basic diet of grain) and a low rate of hypertensive complications of pregnancy. Belizan's group followed up these observations with a

small randomized controlled trial in which 36 pregnant women aged between 20 and 35 were allocated to 1g calcium daily, 2g calcium daily or placebo from 15 weeks of pregnancy until delivery. SBP fell by a greater amount in both supplemented groups during the second trimester of pregnancy with respect to the control group; by the third trimester values for the 1g calcium group approached control values but the 2g group remained lower. DBP fell during the second trimester in all three groups but the fall was greater in both supplemented groups who had similar values. By the third trimester the unsupplemented women had the highest DBPs. PTH levels were measured during this study and found to be slightly, but not significantly, lower in the 2g group (Belizan, Villar, Zalazar, Rojas, Chan & Bryce, 1983[a]). A much larger trial from the same group studied 1194 nulliparous women who received either 2g of calcium or placebo from 20 weeks of pregnancy until delivery; the incidence of hypertensive complications of pregnancy was significantly reduced in the supplemented group (Belizan, Villar, Gonzalez, Campodonico & Bergei, 1991). Other studies have confirmed these findings (Villar, Repke, Belizan & Pareja, 1987 and Lopez-Jaramillo, Narvaez, Weigel & Yepez, 1989).

In 1988, Belizan, Villar and Repke suggested a mechanism whereby a low calcium diet resulted in increased PTH secretion leading to a rise in intracellular ionized calcium with increased muscular reactivity in vascular smooth muscle and hence to an increase in blood pressure (Fig 1.2iii).

$\downarrow$Ca (diet) $\rightarrow$ $\uparrow$PTH $\rightarrow$ $\uparrow$cytosolic iCa^{2+} $\rightarrow$ $\uparrow$vascular reactivity $\rightarrow$ $\uparrow$BP

$\uparrow$Ca (diet) $\rightarrow$ $\downarrow$ PTH $\rightarrow$ $\downarrow$ cytosolic iCa^{2+} $\rightarrow$ $\downarrow$vascular reactivity $\rightarrow$ $\downarrow$BP

Fig 1.2iii Hypothetical steps of the effect of calcium intake on blood pressure mediated by PTH (after Belizan, Villar & Repke, 1988)

A different mechanism to explain the BP lowering effect of calcium was suggested by Lopez-Jaramillo *et al* (1989) who postulated that calcium intake may affect the synthesis of prostaglandin I_2 (PGI_2), which is a potent vasodilator, but they did not measure this in their study.

In summary therefore most of the earlier published clinical trials on calcium supplementation and blood pressure during pregnancy agreed that calcium is associated with a lower incidence of hypertension. However these trials were conducted in regions where the normal dietary intake of calcium is very low compared to developed countries. Early work also only looked at small numbers of women - 36 in Belizan's original trial in 1983[b], 52 in Villar's trial of 1987 and 106 (Lopez-Jaramillo *et al*, 1989). Therefore further larger trials were established. Four years after work on this thesis began the results of the largest of these, that run by the National Institute of Child Health and Human Development in the USA, which recruited 4 589 nulliparous woman who were randomized to either 2g calcium or placebo daily from the second trimester until delivery, reported no beneficial effect of calcium supplementation on BP. These woman were already taking a Western diet containing adequate calcium (Levine, Hauth, Curet, Sibai, Catalano, Morris, DerSimonian, Esterlitz, Raymond, Bild, Clemens & Cutler, 1997). Two years later the Australian group reported their results which did suggest a beneficial effect of calcium supplements in reducing the incidence of PE, defined as one DBP reading of $\geq$ 110mmHG or two DBP readings of $\geq$ 90mmHg together with 2+ proteinuria or $\geq$ 300mg proteinuria/day. Their results showed a relative risk of 0.44[95% CI 0.21-0.90; p=0.02] for developing PE in the calcium group. However it should be noted that the initial power calculations for this trial suggested that 948 nulliparous women needed to be recruited but, due to a lack of funding, the study was stopped after only 456 women had been enrolled (Crowther, Hiller, Pridmore, Bryce, Duggan, Hague, & Robinson, 1999).

1.2K Calcium metabolism in insulin dependent diabetes mellitus

Since the early 1970s, when it was first suggested that osteoporotic fractures might occur more frequently in diabetic subjects, evidence has been accumulating for abnormal calcium metabolism in IDDM. McNair, Madsbad, Christiansen, Faber, Transbol & Binder (1978[a]) showed that bone mineral mass was reduced by about 10% in IDDM individuals aged from 7-70 years. They also found that the initiation of osteopenia seemed to coincide with the onset of clinical IDDM and significantly reduced ($p<0.001$) bone mineral content was seen after two years of disease duration.

In the following year the same group reported high PO_4 concentrations in IDDM subjects (McNair *et al,* 1979). Later it was found that IDDM subjects had reduced levels of serum iCa^{2+} compared with normal controls; this reduction in serum iCa^{2+} was most marked in IDDM children under 16 (Fogh-Anderson *et al,* 1983). However the data are not all in agreement with Witt, White, Santiago, Seino & Avioli, (1983) reporting normal levels of serum iCa^{2+} in IDDM children although these were not directly compared with control children. Several studies (summarized by McNair in 1988) have also shown lower levels of serum Mg in IDDM probably caused by inhibited reabsorption of Mg in the DCT where glucose is being preferentially reabsorbed (Lindeman *et al,* 1967) - see above.

There are several reasons why iCa^{2+} may be low in IDDM subjects. Levels of lactate, beta-hydroxybutyrate and acetoacetate are increased and thus more calcium will be bound to these anions; also levels of non-esterified fatty acids are raised (Hansen & Johansen, 1970) and this will increase the amount of calcium bound to albumin. The normal response to a lowered iCa^{2+} is an increase in iPTH secretion, however this appears to be impaired in IDDM. In 1983 Fogh-Anderson *et al,* and another group (Witt *et al,* 1983) reported low PTH (using a C-terminal immunoassay) despite low concentrations of iCa^{2+} in IDDM subjects. Subsequent work using assays for iPTH have confirmed this (Sagegese, Bertelloni, Baroncelli, Federico, Calisti & Fusaro, 1988; Thalassinos *et al,* 1993). The latter group also showed an increase, although not to normal levels, in IDDM subjects in whom glycaemic control improved. It was mentioned above (section 1.2C) that Mg deficiency impairs PTH secretion and this may be the mechanism behind the low values of iPTH found in IDDM subjects as Mg excretion has been found to be increased in both diabetic rats (Fort, Lifshitz, Wapnir & Wapnir, 1977) and humans (McNair *et al,* 1979).

Calcium excretion is also raised in IDDM subjects compared to normal controls (McNair *et al,* 1979; Witt *et al,* 1983). This renal wastage of calcium is likely to be caused by both an osmotic diuresis produced by hyperglycaemia and also the low levels of iPTH. Improving glycaemic control leads to both a reduction in urinary calcium excretion and a rise in iPTH levels (Thalassinos, Hadjiyanni, Tzanela, Alevizaki & Philokiprou, 1993).

Intestinal absorption of calcium has been shown to be reduced in diabetic compared to normal rats (Schneider & Schedl, 1972). The authors suggested that the low levels of PTH in diabetic rats might result in decreased conversion of 25-HCC to the more active 1,25-DHCC and hence to impaired intestinal calcium uptake; certainly administration of 1,25-DHCC to rats improved calcium absorption (Schneider, Omdahl & Schedl, 1976). However studies on human diabetic subjects have not found decreased absorption of dietary calcium but rather *hyper*absorption (Witt *et al,* 1983).

Concentrations of vitamin D metabolites may be altered in IDDM although the literature is confusing. In 1982 Christiansen, Christensen, McNair, Nielsen, & Madsbad compared 26 male IDDM subjects with normal controls and found decreased 25-HCC, very reduced 24,25-DHCC but virtually normal 1,25-DHCC in the IDDM group. Later Witt *et al,* 1983 also reported similar levels of 1,25-DHCC in young IDDM subjects compared with controls bur raised levels of 24,25-DHCC; however studies on vitamin D and bone metabolism in children who are still actively growing cannot be directly extrapolated to the adult population. Further studies on adult, Caucasian IDDM subjects and controls found no differences in 25-HCC or 1,25-DHCC levels (Nyomba, Bouillon, Bidingija, Kandjingu & de Moor, 1986).

The differing levels of 1,25-DHCC found in diabetic, compared to normal, subjects may reflect differences in glycaemic control leading to renal damage and hence to reduced renal-1-hydroxylase activity. Certainly Nyomba *et al* (1986) reported low 1,25-DHCC in poorly controlled IDDM Bantu subjects. Similarly low levels have been found in adolescent IDDM subjects with persistent microalbuminuria compared to those with no microalbuminuria (Verrotti, Basciani, Carle, Morgese & Chiarelli, 1999) and in NIDDM patients with early diabetic nephropathy (Inukai, Fujiwara, Tayama, Aso & Takemura, 1997).

Calcitonin concentrations are raised in IDDM (Schaerstrom, Hamfelt & Soderhjelm, 1986; Blasiak, 1989). In the face of low iPTH this may help to maintain serum iCa^{2+} within normal limits but this is still speculative.

1.2L Calcium metabolism in IDDM pregnancy

In general very little is known about this subject although calcium metabolism in the infants of diabetic mothers has been quite extensively studied and indeed this was one of the reasons for embarking on the prospective study described in this thesis.

An increased rate of neonatal tetany, associated with low serum concentrations of calcium, were first reported in the infants of IDDM mothers by Craig in 1958, however half of these infants were delivered before 37 weeks gestation. In order to see if this neonatal hypocalcaemia was associated with maternal diabetes rather than prematurity Tsang, Kleinman, Sutherland & Light, (1972) looked at infants of diabetic mothers compared with control infants matched for gestational age and confirmed that the infants of the diabetic mothers were significantly more hypocalcaemic. They suggested that relative maternal hyperparathyroidism in diabetic pregnancy led to fetal hypoparathyroidism and hence low serum calcium but they did not measure PTH in diabetic pregnancy. Using a C-terminal assay for PTH Cruikshank, Pitkin, Varner, Williams & Hargis (1983) reported reduced concentrations of PTH in IDDM pregnancy and a later study looking at iPTH (Mimouni *et al,* 1989) found low-normal concentrations of iPTH in IDDM pregnancy compared to normal pregnancy. Although there is no evidence to support maternal hyperparathyroidism leading to neonatal hypoparathyroidism infants of diabetic women do seem to be hypoparathyroid (Bergman, Kjellmer & Selstam, 1974; Tsang, Wen-Chen, Friedman, Gigger, Steichen, Koffler, Fenton, Brown, Pramanik, Keenan, Strub & Joyce, 1975; Noguchi, Eren & Tsang, 1980; Cruikshank *et al,* 1983).

Impaired maternal Mg metabolism in IDDM pregnancy has been suggested as the mechanism for neonatal hypoparathyroidism and hypocalcaemia and this hypothesis is better supported by experimental evidence. IDDM mothers are significantly hypomagnesaemic compared to normal pregnant women (Mimouni *et al,* 1989). Maternal hypomagnesaemia in pregnant diabetic women is correlated with low levels of serum Mg in their infants (Tsang, Strub, Brown, Steichen, Hartman & Wen-Chen, 1976) and as mentioned above (section 1.2C, p14) low levels of Mg are associated with impaired release of PTH.

1.3 THE RENIN-ANGIOTENSIN SYSTEM

Since 1898 when Tigerstedt and Bergman first extracted the protein substance which they named "renin" from the renal cortex of rabbits and demonstrated that it had a pressor effect when injected intravascularly, a massive amount of observation and experimentation has been carried out on what is now known as "the renin-angiotensin system" (RAS). Renin has been shown to be an enzyme which in itself does not produce hypertension; instead it acts on the substrate angiotensinogen (Aogen) in the first reaction of a cascade which ultimately produces angiotensin II (Ang II), the most potent circulating vasoconstrictor hormone yet described, - see Fig 1.3ii. Renin and Ang II are now known to be distributed throughout the body and throughout vertebrate species (Robertson, 1993). Ang II has been shown to have other actions besides vasoconstriction; for example it regulates aldosterone production, is a mediator in the control of thirst (Fitzsimons, 1993) and is involved in reproductive steroidogenesis (Lumbers, 1993, Inagami, 1993). Much recent work has also focused on its role as an angiogenic agent and as a general growth promoter in many other tissues (Huckle & Earp, 1994).

1.3A Components of the RAS

In non-pregnant mammals most renin is synthesized within the kidney, in the juxtaglomerular (JG) apparatus, which is located mainly within the afferent arteriole walls (fig.1.3i). Following the discovery of renin it became apparent that most circulating renin is in an inactive, prohormone, form known as 'prorenin' first described by Lumbers in 1971, and present at ten times the concentration of renin in plasma (Sealey, Atlas & Laragh 1980). Prorenin protein contains an amino-terminal peptide of 43 amino acids split off by a proteolytic enzyme to create fully active renin (Schalekamp & Derkx, 1993) (fig 1.3ii). The renin molecule is composed of two very similar domains separated by a deep cleft which contains the binding site for renin substrate or Aogen (Murakumi, 1984). This conversion of prorenin to renin can occur extrarenally as well as in the JG apparatus.

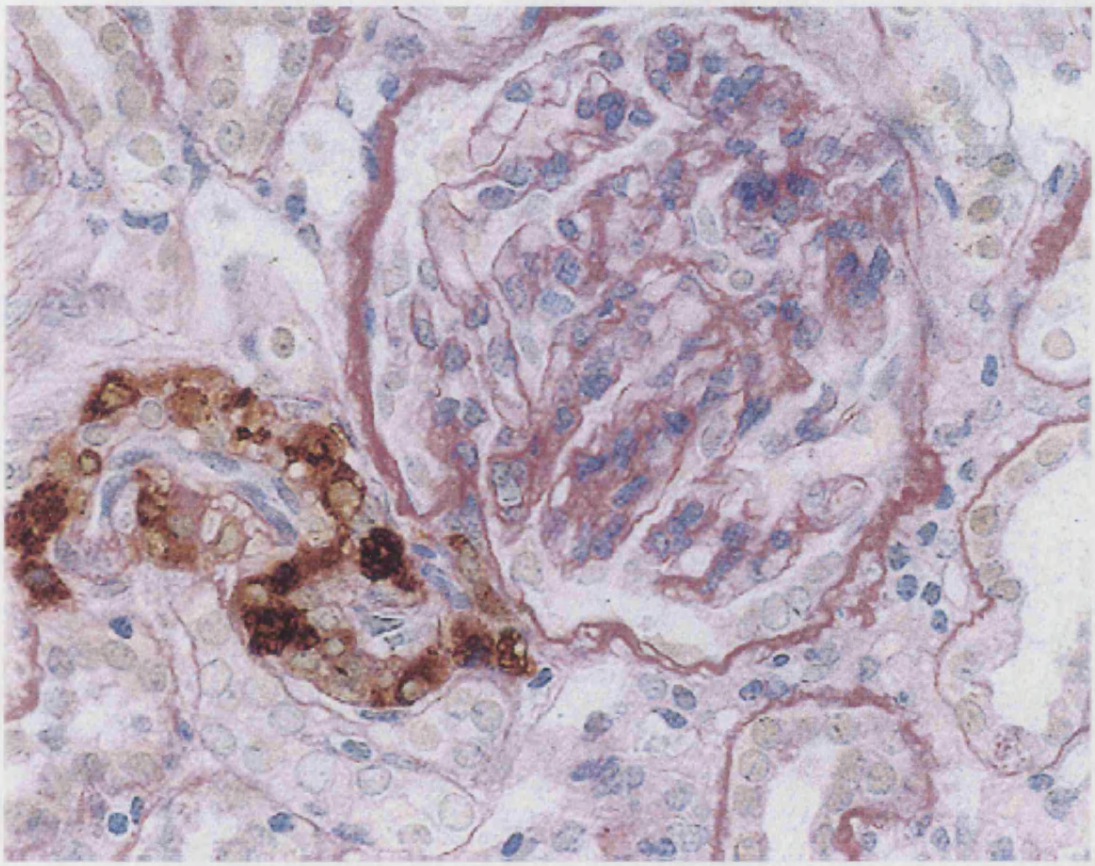


Fig 1.3i A juxtaglomerular apparatus showing many renin-secreting cells (stained brown) extending in continuity down the afferent arteriole.

Renin peroxiase-antiperoxidase and periodic acid/Schiff stain. (Robertson, 1993)

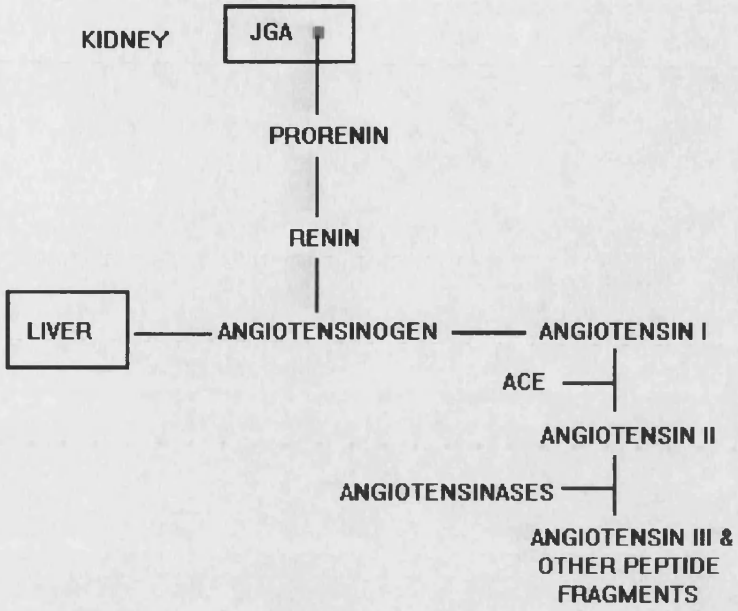


Fig 1.3ii A simplified overview of the components of the RAS

Aogen, is an α_2-globulin synthesized in and secreted from the liver into the plasma. It exists in several different forms, especially in pregnancy, all of which arise from a single precursor of molecular weight approximately 50,000. Renin splits off a decapeptide, Ang I, from Aogen and Ang I is subsequently cleaved by angiotensin converting enzyme (ACE) to produce Ang II, the major active component of the whole RAS (fig. 1.3iii). Ang II is then broken down by various angiotensinases to form the less active angiotensin III (Ang III) and other fragments (fig. 1.3ii).

H - Asp - Arg - Val - Tyr - Ile - His - Pro - Phe -OH

Fig. 1.3iii The structure of human Ang II

1.3B Measurement of renin and angiotensin

Plasma renin activity (PRA) is the ability of the endogenous active renin in a plasma sample, acting on endogenous Aogen to generate Ang I under physiological

temperature and pH conditions. This enzymatic reaction remains the most commonly used method to quantify PRA although some groups now use a specific direct immunoassay (see below). The plasma renin concentration (PRC) is measured by reacting the renin in a sample with excess (heterologous) Aogen under the same conditions. Plasma Aogen is measured by its ability to generate Ang I from an excess of homologous renin, again under the same physiological conditions.

The development of radioimmunoassays to measure components of the RAS has been difficult because of problems with antibody specificity. Some initial radioimmunoassays gave lower plasma renin substrate (PRS) values than enzyme methods, presumably because Aogen exists in several forms not all of which were being detected by the assay; other assays produced higher values and were thought to be measuring immunoreactive (but not biologically active) degradation products as well as pure Aogen. Since Aogen is a large molecule from which only the first ten amino acids are cleaved to form Ang I, antibodies need to be directed specifically at this decapeptide to avoid misleading results. Using monoclonal antibodies directed against this portion of Aogen an enzyme-linked immunosorbent assay has now been developed.

As the PRA alters under different clinical conditions, for example it is high during treatment with ACE inhibitors because of the lack of negative feedback of Ang II on renin production, it is useful to measure levels of Ang II, the active end-product of the RAS. This is difficult because physiological concentrations of Ang II are usually in the low picomolar range and require very sensitive assays. Also there are precursor and breakdown peptides which can cross-react in radioimmunoassays. Modern assays now obtain specificity by isolating Ang II from other peptides by high performance liquid chromatography or ion-exchange chromatography, and sensitivity by using radioimmunoassay techniques. Sample collection is also important as Ang II can be generated in plasma after sampling if inhibitors of cleavage are not used and samples are neither analysed immediately nor frozen. Normal diurnal variation must also be allowed for as should subject position and time from the last meal (Nussberger & Brunner, 1993).

Although PRA is widely measured in clinical practice in non-pregnant subjects it was not measured in this thesis because in pregnancy Aogen concentrations are rate-limiting (see below). Instead enzyme and substrate concentrations were measured separately to obtain a better idea of what was acting where within the cascade.

1.3C Control of renin release

The physiological stimulants to renin release reflect its crucial role in maintaining blood pressure and circulating volume. Five basic mechanisms have been described: intrarenal baroreceptors, the amount of sodium sensed by the macula densa segment of the distal convoluted tubule (DCT), the sympathetic nervous system and humorally released catecholamines, other hormonal factors (eg. Ang II, prostaglandins and steroids) and plasma electrolytes such as potassium and Ca (Keeton & Campbell, 1981).

i) Baroreceptors

The existence of a renal baroreceptor that either increased or decreased renin secretion in response to a decrease or increase in mean renal perfusion was first suggested by Tobian in 1959. Since then the concept has been extensively investigated and refined and, in summary, it is now thought that these baroreceptors are located in the afferent renal glomerular arteriole. Stimuli which lead to a decrease in the transmural pressure gradient of these receptors, eg. a fall in blood pressure secondary to haemorrhage, lead to increased renin secretion.

ii) The macula densa

The macula densa region of the DCT is composed of a specialized group of columnar or cuboidal cells that are in close contact with the granular juxtaglomerular cells of the glomerulus from which the tubule originates. The macula densa is usually found at the boundary of the ascending loop of Henle and the DCT and electron microscopy of this region has shown that the macula densa and the granular cells are only separated by a thin, incomplete basement membrane. Much experimental work has been done on the role of the macula densa in renin release and this is summarized by Keeton & Campbell

(1981); in essence a decrease in sodium transport to the macula densa stimulates renin release in humans. In contrast, in the rat, chloride seems to be the ion involved in the regulation of renin release by the macula densa and this illustrates the difficulty of extrapolating from animal work to humans.

iii) The sympathetic nervous system

Activation of β-adrenergic neurones of the sympathetic nervous system within the JG cells stimulates renin release. This can be demonstrated by the intrarenal injection of both adrenaline and noradrenalin, and by the inhibition of renin release by prior administration of propranolol. Also the plasma renin activity (PRA) is often elevated in the hypertension associated with phaeochromocytoma (Hiner, Gruskin, Baluarte, Cote, Sapire & Levitsky, 1976). Physiologically the sympathetic activity generated in response to hypovolaemic shock will result in increased renin secretion which acts both to maintain blood pressure and, via aldosterone, to conserve sodium and hence increase circulating volume.

iv) Hormonal and humoral factors

Various hormones influence renin secretion. Most importantly there is a short-loop negative feedback by plasma Ang II itself, suppression by anti-diuretic hormone and stimulation by prostaglandins (PG) and prostacyclin.

The relationship between PGs and renin release was first studied in 1968 by Vander who reported inconsistent results of infused PGE_1 and PGE_2 on PRA in dogs. One reason for these inconsistencies was that data from several doses of the different prostaglandins were pooled. Later work found a consistent rise in PRA when PGE_1 was injected into dogs (Werning, Vetter, Weidemann, Schwiekert, Stiel & Siegenthaler, 1971). Further work in humans showed stimulation of renin release by PGA_1 (Golub, Speckart, Zia & Horton, 1976). In the mid-1970s prostacylin was discovered and as this was soon shown to be more potent than PGs of the E and F series the focus of research shifted to the new compound. There is now compelling evidence that prostacyclin has a direct stimulatory effect on renin release in humans,

rather than an indirect effect via its vasodilatory and hypotensive actions (Patrono, Pugliese & Ciabbattoni, 1982).

Oestrogens also alter renin secretion. It has been observed that PRA is increased during the luteal phase of the human menstrual cycle (Brown, Davies, Lever & Robertson 1964[a]) and during human pregnancy (Brown, Davies, Lever & Robertson 1964[b]). As discussed below PRA is a measure which is dependent on both enzyme and substrate concentration; the rise in PRA during the luteal phase is almost entirely due to the stimulatory effect of oestrogen on Aogen synthesis rather than on renin synthesis itself. Progesterone can also stimulate renin release but this is thought to be secondary to the induction of a natruresis brought about by the similarity between the progesterone and aldosterone molecules.

In view of the above it would be expected that oral contraceptives would alter renin release. Early work in this field was performed by Skinner and colleagues (1969). They found a two-fold increase in PRA and PRS concentration together with a suppression of PRC in women taking combined oral contraceptives (COC). They concluded that COCs increased PRA by increasing blood PRS and they postulated that increased levels of Ang II produced a fall in PRC by negative feedback. Subsequent work has confirmed the rise in PRS produced by the COC but has not shown an increase in PRA (Derkx, Stuenkel, Schalekamp, Visser, Huisveld & Schalekamp, 1986). These differences may be due to the lower oestrogen doses now used in oral contraceptives. Possibly also, inadvertent activation of prorenin occured in earlier studies which used acid-pretreated plasma, as it was not until 1971 that it was realised that acid pre-treatment of samples activated a form of renin (ie. prorenin) not active at physiological pH (Lumbers, 1971).

v) Plasma electrolytes

Numerous electrolytes have been shown to alter renin release and these have been reviewed by Keeton & Campbell (1981). The decrease in renin secretion caused by an increased load of sodium at the macula densa has already been mentioned. Potassium also appears to decrease renin release while an increase in plasma magnesium stimulates renin release. Conversely falling magnesium levels reduce renin production.

Calcium and parathyroid hormone

iCa^{2+} is known to be required by most secretory cells for stimulus-secretion coupling to occur (Rubin, 1970). It is also involved in the contraction of smooth muscle (see below). Since the granular JG cells are both secretory cells and modified smooth muscle cells one would expect calcium to be involved in the control of renin release. However the relationship is likely to be even more complicated since calcium can inhibit sodium reabsorption (Freedman, Moulton & Spencer, 1958), alter catecholamine release (Rubin, 1970) and affect blood pressure (see below).

Early experiments showed that the infusion of both calcium chloride and calcium gluconate into anaesthetized dogs reduced the rate of renin secretion (Kotchen, Maull, Luke, Rees & Flamenbaum, 1974; Watkins, Davis, Lohmeier & Freeman, 1976). This inhibition of renin release was thought to occur via a direct action of iCa^{2+} on the JG cells, not via the renal nerves where increasing iCa^{2+} would cause an increase in noradrenaline secretion and hence an increase in renin secretion, and not by an action on the baroreceptors where a decrease in afferent arteriolar radius, in the face of a constant renal perfusion pressure, would also lead to increased renin secretion.

Numerous further experiments have increased the evidence in favour of calcium having an inhibitory effect on renin release, in contrast to the more typical findings in exocrine and endocrine cells, where the calcium concentration is usually positively correlated with the secretion rate. Following the above experiments which involved either whole animals or slices of renal cortex Baumbach & Leyssac (1977) looked at isolated preparations of rat glomeruli. They found that decreasing the extracellular concentration of iCa^{2+} increasd renin secretion, while increasing the intracellular concentration of iCa^{2+} using a calcium ionophore which facilitated iCa^{2+} influx into the cells inhibited renin release. They also showed that the calcium antagonist lanthanum decreased renin release. Others found that chelating extracellular iCa^{2+} with ethylenediamintetraacetic acid (EDTA) which subsequently produced a fall in intracellular iCa^{2+}, increased renin release (Park & Malvin, 1978). Much more recently Porter, Conlin, Scott, Brown & El-Hajj Fuleihan (1999) showed that physiological increases in serum iCa^{2+} in humans were associated with an increase in PRA. The mechanism(s) by which raised intracellular iCa^{2+} concentrations inhibit renin release

remains hypothetical and two are outlined in a review by Skϕtt & Jensen (1993). One possibility is that iCa^{2+} may activate chloride channels allowing chloride to flow out of cells [followed by potassium], thus the osmotic pressure of the cell falls and water is lost by the JG cell and its renin-containing organelles which therefore are less able to secrete renin; another hypothesis is that the secretory granules and the cell membrane are separated by a network of myofilaments which contract as the intracellular concentration of $iCa^{2+}$is raised, this decreases contact between them and so secretion cannot occur.

Chronic calcium loading has been shown not to affect PRA, or renal renin content in rats (Kotchen *et al*, 1974; Kotchen, Galla & Luke, 1977). In 1975 Brinton reported elevated PRA in four out of seven hypertensive patients with primary hyperparathyroidism but this was not the case with normotensive patients with primary hyperparathyroidism or secondary hyperparathyroidism or with hypercalcaemia of other cause. These authors concluded that PTH *per se* had no affect on renin secretion. Later work in dogs (Smith, Mouw & Vander, 1979) showed that the infusion of relatively large doses of bovine PTH did lead to an increase in PRA which fell rapidly once the infusion was stopped. The same authors also stimulated the production of endogenous PTH, in dogs, by infusing citrate into the carotid artery and again found an increase in PRA in some, but not all, animals. It was noted that the non-responders had the largest increases in sodium excretion suggesting that PTH may exert two opposing effects on renin secretion; indirectly by inhibiting proximal sodium reabsorption it may increase the macula densa sodium load and thereby inhibit renin secretion, while it also directly stimulates renin release.

In 1992 Grant, Mandel, Brown, Williams & Seely showed that infusion of the 1-34 amino-terminal fragment of PTH increased PRA by over two-fold. The following year a French group (Saussine, Judes, Massfelder, Musso, Simeoni, Hannedouche & Helwig, 1993) provided evidence for a direct stimulatory effect of PTH(1-34) on renin secretion in isolated rat kidneys. They then went on to look at the effects of extracellular iCa^{2+} alone and PTH and extracellular iCa^{2+} combined on renin release in isolated preparations of rabbit glomeruli. They found that a low iCa^{2+} medium

increased basal renin secretion whilst high concentrations of extracellular iCa^{2+} inhibited renin secretion. However PTH was only able to increase renin production in the presence of extracellular iCa^{2+}. They concluded that PTH was acting by inhibiting calcium influx to the glomerular cells, thus reducing intracellular iCa^{2+} and stimulating renin release as described above.

1.3D The pharmacokinetics of renin production

The reaction of renin on Aogen to produce Ang I is dependent on both the concentration of enzyme (renin) and substrate (Aogen) in the plasma and obeys Michaelis-Menton first order kinetics. In men and non-pregnant women the concentration of Aogen is around the K_M for the reaction meaning that Ang I is generated at about half the maximal velocity possible. In pregnancy Aogen concentrations rise with gestation, peaking at term (Skinner, Lumbers & Symonds 1972, Baker, Broughton Pipkin & Symonds 1990). The production of angiotensin during pregnancy is known to be substrate dependent (Skinner *et al*, 1972), therefore the rise in Aogen alone is enough to increase the rate of Ang II production. Ang II levels rise significantly by the second trimester of human pregnancy compared with non-pregnant women (Baker *et al*, 1990). In the human fetus Aogen levels are less that those of maternal levels although slightly higher than in non-pregnant adults (Tetlow & Broughton Pipkin, 1983). It seems that in the fetus, like the pregnant mother, it is the Aogen, rather than the renin, concentration which is rate-limiting in the reaction (Skinner *et al*, 1972).

Once, all ACE activity was thought to occur in the lung but now it is known that ACE is synthesised by the vascular endothelial cells of most tissues (Miyazaki, Okunishi, Nishimura & Toda, 1984). For example, intrarenal Ang II is synthesised by ACE produced in the endothelium of the proximal tubular cells (Chai, Allen & Adam, 1986). The high vascularization of the lung probably explains why the bulk of conversion of Ang I to Ang II takes place there. The placenta is also highly vascularized and

therefore another important site of conversion; at term ACE activity in the placenta is about 13% of the adult lung value (Sim & Seng, 1984).

Ang II has a very short halflife being broken down by various angiotensinase enzymes to peptide fragments. For example angiotensinase A, found in the brain, kidney and vascular endothelium splits off the aspartate from position 1 creating the heptapeptide Ang III a less potent vasoconstrictor than Ang II (Harding & Felix, 1987). Ang III is hydrolysed even more rapidly than Ang II so its duration of action is much shorter. An endopeptidase (angiotensinase C) creates a heptapeptide angiotensin-(1-7), once thought to be inactive but now been known to bind to receptors in the central nervous system, (Ferrario, Barnes, Block, Brosnihan, Diz, Khosla & Santos, 1990) (section 1.3F, p44).

1.3E Angiotensin receptors

Ang II acts on two receptors - the AT1 and AT2 receptors. The AT1 receptors mediate most of the "classical" actions of Ang II such as vasoconstriction. Ang II binds to the transmembrane AT1 receptor which contains a guanine nucleotide regulatory protein (G-protein) which is coupled to phospholipase C. Binding of the Ang II activates the phospholipase cascade releasing second messengers such as inositol (1,4,5) triphosphate which then releases iCa^{2+} from the endoplasmic reticulum. This causes a rise in intracellular free iCa^{2+} which acts as a further messenger; in vascular smooth muscle; the increased iCa^{2+} concentration will also lead directly to vasoconstriction by initiating the actin-myosin interaction (fig. 1.3iv).

Within the kidney AT1 receptors (Edwards, Stack, Weidley, Aiyar, Kennan, Hill & Weinstock 1992; and Sechi, Grady, Griffin, Kalinyak & Schambelan 1992) are situated on the afferent and efferent glomerular arterioles but stimulation of them preferentially causes efferent vasoconstriction. If renal blood flow is reduced by generalized hypotension this efferent vasoconstriction will help to maintain the glomerular filtration pressure. AT1 receptors are inhibited by losartan, valsartan, ibresartan and candesartan and these antagonists are now licensed for clinical use in the treatment of essential hypertension (fig. 1.3iv).

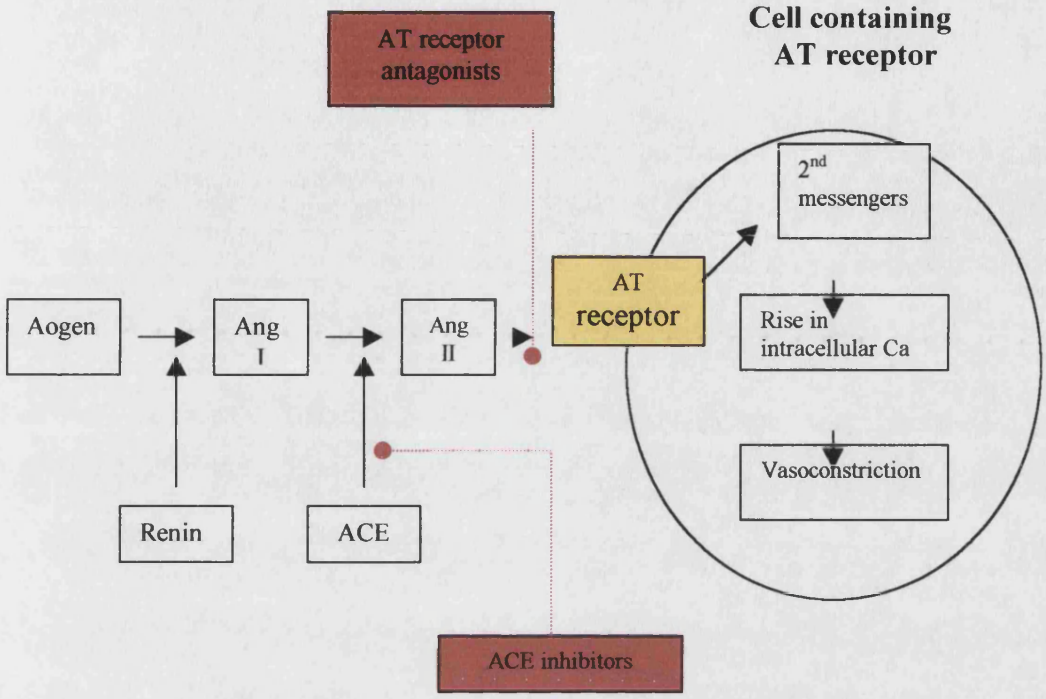


Fig. 1.3iv The place of AT receptors and their inhibitors within the RAS

AT2 receptors are not yet fully characterized; they are found in the reproductive tract, some cerebral vessels and the kidney and may be concerned with growth (see below). AT2 receptors do not seem to be coupled to the mobilization of intracellular Ca^{2+} as AT1 receptors are (Chappell, Jacobsen & Tallant,1995). Within the kidney Ang II acting on AT2 receptors stimulates sodium and fluid reabsorption by a direct effect on the renal tubules which tends to restore circulating volume (Goldfarb, 1994).

1.3F Extrarenal actions of the RAS

i) Aldosterone secretion

The first non-vasoconstrictor effect of the RAS to be described was the control of aldosterone secretion. Initially pituitary adrenocorticotrophic (ACTH) hormone was

thought to regulate this, but in 1961 Davis and colleagues reported that nephrectomy abolished the aldosterone response to acute haemorrhage in hypophysectomized dogs, and that giving synthetic Ang II markedly increased aldosterone secretion; Ang III has a similar effect in animals but not in man. It is also known clinically that in hypoadrenal patients whose disease has an adrenal origin fludrocortisone is needed in approximately 90% whereas in those with hypopituitary hypoadrenalism it is needed in only 10%.

Aldosterone increases sodium absorption from urine, sweat, saliva and the gastrointestinal secretions. The urinary reabsorption of sodium is followed by water reabsorption in the distal collecting tubule (DCT) and collecting duct. At the same time that sodium is reabsorbed potassium and hydrogen ions are secreted so the extracellular fluid (ECF) volume is increased while acid-base balance is maintained. Thus renin acts to maintain blood pressure in the short term by causing rapid vasoconstriction and in the longer term by increasing circulating volume via aldosterone (Espiner & Nicholls,1993) and also by stimulating drinking (see below).

ii) The RAS within the brain

The first central action of Ang II was discovered by Bickerton and Buckley in 1961 when they showed that Ang II injected centrally, into the carotid artery, could raise blood pressure in vascularly isolated dogs' heads connected to the body by the nervous system only. This pressor effect is mediated by the sympathetic nervous system and can be reduced by α-adrenergic blockers.

The next central action of the RAS to be demonstrated was its involvement in the control of thirst and sodium appetite. High levels of Ang II-responsive neurones have been found in the subfornical organ and in the vascular organ of the lamina terminalis which control drinking and salt intake. Essentially raising the intracerebral levels of Ang II leads to increased drinking and salt intake; this of course will act to restore circulating fluid volume after fluid loss so once more Ang II is seen to be maintaining blood pressure (Fitzsimons, 1993).

Recent evidence suggests that Ang II is not the only component of the RAS which is physiologically active in the brain. Ferrario *et al* (1990) have shown that a prolyl-endopeptidase together with other endopeptidases act centrally to produce angiotensin-(1-7) directly from Ang I without involving ACE. They next demonstrated that angiotensin-(1-7) was as potent as Ang II in stimulating release of vasopressin from the posterior pituitary gland but that it lacked direct pressor and dipsogenic effects.

iii) Ang II in the ovary and testis

Renin and Ang II have been shown to be produced locally within both the ovary and testis where they have a paracrine role. Follicular aspirates from stimulated ovarian follicles were shown to contain fourteen times as much renin-like activity as plasma (Fernandez, Tarlatz, Rzasa, Caride, Laufer, Negro-Vilar, DeCherney & Naftolin 1985). Subsequently follicular renin concentration (FRC) was shown to be similar to PRC in unstimulated cycles; FRC was unaffected by clomiphene citrate but increased when gonadotrophins were used for ovarian hyperstimulation. Follicular renin substrate (FRS) was only slightly higher than PRS and barely increased with hyperstimulation. The authors concluded from this that gonadotrophins themselves stimulate renin production and that within the ovary it is FRS which is the rate-limiting factor in the generation of Ang I (Brameld, Broughton Pipkin & Symonds, 1990). Growth and maturation of the ovarian follicle requires a high blood flow and Ang II probably plays an intraovarian angiogenic role - see below. It also appears to stimulate granulosa cell aromatase activity which leads to oestradiol production. Its vasoconstrictor activities may also be involved in non-dominant follicular atresia (Lumbers, 1993).

In the testis Ang II is one of the mediators of seminiferous tubule contraction and it also regulates fluid and anion secretion into the epididymal fluid, both of which activities contribute to the transport of sperm. Whether renin or Ang II are involved in male steroidogenesis is not definitely known, but it is possible Ang II is a moderate inhibitor of testosterone production (Inagami, 1993).

1.3G Angiotensin II as a regulator of cell growth and proliferation

In 1977 it was reported that Ang II could promote the proliferation of adrenocortical cells in culture (Gil, Ill & Simonian, 1977) and then the same effect was noted with fibroblasts (Schelling, Ganten, Speck & Fischer, 1979). Mention has been made above of the widespread occurrence of ACE activity, once thought to be limited to the lung, and it appears that these locally-regulated pathways for Ang II production, which are of course independent of the systemic physiological factors regulating vasomotor tone, are involved in growth regulation.

This action of Ang II seems to be mediated via AT 1 receptors. Binding to these by Ang II activates tyrosine kinases, a response which is dependent on iCa^{2+}. This iCa^{2+}-dependent, Ang II-stimulated tyrosine phosphorylation leads to the activation of several peptides known to be involved in cell growth such as platelet derived growth factor, basic fibroblast growth factor and transforming growth factor β_1.

In addition to stimulating the production of these factors Ang II also appears to influence the expression of many growth-related genes such as c-*fos*, c-*jun*, c-*myc*, *JunB*, *Egr-1* and *cMGI*, known to be active in adrenal tissue, hepatocytes, mesangial cells, heart, intestinal epithelial cells and the brain (Huckle & Earp, 1994). Whether these growth-promoting effects of Ang II are important physiologically, in the non-pregnant subject, is not yet established. They do seem to have a pathogenic role. For example the left ventricular hypertrophy which occurs as an adaptive process in chronic hypertension is Ang II mediated and this can be reversed (Asmar, Pannier, Santoni, Laurent, London, Levy & Safar 1988; Gilbert 1992) or prevented, (Massie 1992, Kloner & Przyklenk, 1993) by the use of ACE inhibitors, an effect important clinically. This effect of ACE inhibitors occurs at lower concentrations than those needed to produce significant hypotension (Owens 1985; Gohlke, Stoll, Lamberty, Mattfeld, Mall, van Even, Martorana & Unger, 1992). Another pathological condition in which Ang II is implicated as a growth factor is in mesangial proliferation in several of the glomerulopathies (Gavras & Gavras, 1993) where ACE inhibitors are used therapeutically.

1.3H The RAS in insulin dependent diabetes

Circulating Aogen concentrations are normal in both well-controlled (Day, Luetscher & Gonzales, 1975; Feldt-Rasmussen, Mathisen, Deckert, Giese, Christensen, Bent-Hansen & Nielsen, 1987) and unstable (Feriss, O'Hare, Kelleher, Sullivan, Cole, Ross & O'Sullivan, 1985) IDDM. However renin production and secretion by the JG cells of the kidney is abnormal in IDDM. It appears that even in the early stages of IDDM, before there is clinical evidence of nephropathy, more prorenin is secreted whilst levels of active renin are low (Day *et al*, 1975; Luetscher, Kraemer, Wilson, Schwartz & Bryer-Ash, 1985). It is now possible to monitor developing nephropathy using a sensitive assay which is able to detect albumin in the urine at lower concentrations than those detected by albustix, confusingly this is described as microalbuminuria. As nephropathy develops, assessed by measuring microalbuminuria, the levels of prorenin found in both IDDM and NIDDM increase (Luetscher *et al*, 1988). This may be caused by chronic glycosylation, and therefore inactivation, of prorenin-processing enzymes. For example in 1981 Coradello, Pollak, Pagano, Leban & Lubec showed that cathepsin B, a kidney protease that is known to convert prorenin to renin, became inactive after incubation with high concentrations of glucose. Another possibility is that the neuroendocrine processes involved in renin production and secretion are affected by IDDM. In 1979 Tuck, Sambhi & Levin demonstrated decreased sympathetic activity and impaired β-receptor mediated responsiveness of renin release in IDDM patients with peripheral neuropathy. Ultimately as IDDM progresses and JG cells are destroyed by hyalinosis of the renal arterioles there will be less cells available to produce renin.

Since levels of active renin are low one would expect circulating levels of Ang I, Ang II and aldosterone to be low in IDDM and this appears to be the case (Tuck *et al*, 1979; Weidmann, Beretta-Piccoli & Trost, 1985; Feldt-Rasmussen *et al*, 1987).

Serum ACE activity has been variously reported to be increased (Lieberman & Sastre, 1980; Feldt-Rasmussen *et al*, 1987), normal (Giampietro, Lenzi, Sampietro, Miccoli & Navalesi, 1986) or low in diabetic subjects. Discrepant findings may reflect differences

between patient groups studied, control groups or in methodology. Alternatively it may be that any rise in ACE activity in IDDM is intermittent and so not always detectable. However since ACE is normally present in excess the significance of a further rise is unknown.

Different stages of IDDM also have different impacts on the RAS. For example in diabetic ketoacidosis renin, Ang II and aldosterone usually rise acutely whilst ACE activity falls. This activation of the RAS is probably due to the combined effects of sodium, fluid and potassium depletion and raised adrenergic activity. As the ketoacidosis is treated the RAS activity returns to normal (Christlieb, Assal, Katsilambros, Williams, Kosak & Susuki, 1975). As mentioned above as IDDM progresses and complications such as nephropathy develop the RAS is affected increasingly with raised levels of prorenin but reduced active renin.

Hypertension occurs twice as frequently in diabetic patients as in non-diabetics (Christlieb, Warram, Krolewski, Busick, Ganda, Asmal, Soeldner & Bradley, 1981) and it has a significant additive effect on the development and progression of both the macrovascular (atherosclerosis and left ventricular hypertrophy) and microvascular (nephropathy and retinopathy) complications of the disease. All of these complications are a consequence of overgrowth of vascular endothelium and/or smooth muscle and it has been suggested that this overgrowth is Ang II mediated through cytokines and growth factor expression as discussed previously. Certainly ACE inhibitors have been demonstrated to slow the progress of diabetic glomerulosclerosis and atherosclerosis (Hsueh & Anderson, 1993), although these data should be interpreted with caution as it is not entirely clear whether this beneficial effect is produced by inhibiting cell proliferation or the BP lowering effect of ACE inhibitors. Certainly the UK prospective diabetes survey (UKPDS) in non-pregnant type II diabetes mellitus did not find differences between atenolol and captopril in slowing the development of nephropathy. However these results need to be interpreted with caution as the study was not adequately powered to show such diffrerences (UKPDS, 1998). As yet ACE inhibitors have not been shown to affect the progress of retinopathy which is mainly influenced by glycaemic control rather than BP control. Again over the period of the UKPDS patients treated with atenolol and captopril showed an equal deterioration in

retinopathy measured by retinal photography but again this needs to be regarded with caution as mentioned above (UKPDS, 1998).

It seems paradoxical that Ang II may be responsible for this vascular endothelial hypertrophy and renal pathology in IDDM whilst overall levels of both it and Ang I are low in diabetic subjects (see above). In an attempt to reconcile this apparent contradiction, it has been suggested that diabetic patients may have an increased sensitivity to Ang II. In 1984 Drury, Smith & Ferriss demonstrated an increased vasopressor responsiveness to Ang II in type I diabetic patients with no vascular complications and Weidmann *et al,* (1985) showed that the dose of infused Ang II required to increase diastolic pressure by 20mmHg was less in diabetic subjects compared with normal subjects and particularly low in those with diabetes and hypertension. In general, where circulating concentrations of an agonist are low, receptor density for that agonist is increased; this is probably the mechanism for the increased pressor responsiveness to Ang II in IDDM.

The use of ACE inhibitors in diabetic nephropathy

In 1985 Taguma and colleagues reported the results of a study which followed on from the chance observation that proteinuria in one of their long-term haemodialysis patients was reduced after he was given captopril to treat hypertension. They were able to show that proteinuria in 10 diabetic subjects was reduced from 10.6±2.2g/24hr to 6.1±1.4g/24hr ($p<0.01$) following treatment with captopril. This reduction was apparent after 2 weeks of treatment and stabilized within four weeks (Taguma, Kitamoto, Futaki, Ueda, Monma, Ishizaki, Takahashi, Sekino & Sasaki, 1985). Subsequent work from Sweden showed that over two years captopril slowed the rate of decline in glomerular filtration rate in diabetic subjects with nephropathy (Bjorck, Nyberg, Mulec, Granerus, Herlitz & Aurell, 1986).

It was mentioned above that recent results from the UKPDS suggest that this ACE-mediated reduction in development of diabetic nephropathy is merely a side-effect of the BP lowering effect of ACE inhibitors. However in a regression analysis of 100 clinical studies evaluating the effect of various classes of antihypertensive agents on

blood pressure lowering and proteinuria in diabetic nephropathy ACE inhibitors were shown to lower protein excretion more than would be expected if the effect were merely due to the degree of fall of blood pressure which they produce (Keane, Anderson, Aurell, deZeeuw, Nairns & Povar, 1989). One possible mechanism is that overgrowth of the vascular endothelium which leads to glomerulosclerosis, as discussed above, is reduced. Another likely aetiological factor is that intrarenal Ang II causes an increase in the hydraulic pressure across the glomerular capillary membranes, in other words creating "intrarenal hypertension" and that this is reduced by ACE inhibitors; certainly this appears to be the mechanism of angiotensin II-induced proteinuria in rats (Bohrer, Deen, Robertson & Brenner, 1977). However conclusions drawn from meta-analysis of multiple studies (Keane *et al,* 1989) must be regarded with caution and have not received universal agreement.

By analogy with ACE inhibitors, Ang II receptor antagonists may have similar beneficial effects within the kidney. In a small study of patients with non-diabetic proteinuria and hypertension losartan and enalapril reduced proteinuria equally (Gansevoort RT, de Zeeuw D & de Jong PE, 1994). This study was underpowered and further work needs to be done in this area.

1.3J The renin-angiotensin system in pregnancy

The RAS is one of the first hormone systems to recognise pregnancy. In fact plasma concentrations of renin, Aogen, Ang II and aldosterone all change during the menstrual cycle (Brown *et al*, 1964a; Kaulhausen, Leyendecker, Benker & Breuer, 1978) and if pregnancy occurs the raised concentrations found in the luteal phase persist, and indeed increase further.

Cross-sectional and longitudinal studies have looked at PRC, PRS and Ang II concentrations during pregnancy. PRC is significantly increased in the first trimester compared to non-pregnant women and continues to rise throughout pregnancy (Skinner *et al*, 1972, Baker *et al*, 1990) although there may be a small fall at term (Oats, Broughton Pipkin, Symonds & Craven, 1981). PRC falls rapidly after delivery

(Broughton Pipkin, Oats & Symonds, 1978) and is virtually back to non-pregnant levels by 6 weeks postnatally (Baker *et al*, 1990).

The precise mechanism for the hyperreninaemia of pregnancy is not fully understood. It is likely to be progesterone driven as progesterone is know to be a marked natriuretic. This loss of sodium then acts as a stimulus to renin and ultimately aldosterone release and this acts to conserve sodium. Circulating levels of PGE2 and prostacyclin are also elevated during pregnancy and both these, as has already been discussed, increase renin production.

PRS or Aogen rises by about four-fold during pregnancy, with the rise becoming significant by the second trimester (Skinner *et al*, 1972; Weir, Brown, Fraser, Lever, Logan, McIlwaine, Morton & Robertson, 1975; Baker *et al*, 1990). This increase is thought to be oestrogen-driven and similar changes occur in women taking oral contraceptives (Derkx *et al*, 1986). It was mentioned above that Aogen exists in several different forms of different molecular weight. In non-pregnant women the high molecular weight form (HMW-Aogen) makes up less than 5% of the total Aogen. During pregnancy this rises steadily to about 20% by term, the rise becoming statistically significant by the end of the first trimester (Tetlow & Broughton Pipkin 1986). These HMW-Aogen molecules found in pregnancy are likely to be mainly polymers, on the basis of their molecular weights found on gel chromatography Whether or not these HMW-Aogens are physiologically active has not yet been determined.

The increase in both plasma renin and Aogen in normal pregnancy is accompanied by a rise (in the order of 100%), in Ang II (Weir *et al*, 1975; Baker *et al*, 1990) which varies markedly from individual to individual. This rise becomes statistically significant by the second trimester, continues to rise to a maximum in the third trimester, begins to fall within 24 hours of delivery and is virtually back to non-pregnant levels by six weeks postpartum (Baker *et al*, 1990).

In order to prevent a rise in blood pressure in response to these increased levels during pregnancy the maternal vascular system must become less sensitive to the RAS. In

1967 Talledo showed that the vascular response to infused Ang II was significantly reduced in normal pregnancy (Talledo, Rhodes & Livingston, 1967). He then went on to demonstrate increased sensitivity to infused Ang II in pre-eclamptic patients compared to normal pregnant controls (Talledo, Chesley & Zuspan 1968). In 1973 Gant and colleagues reported a prospective study in which they found that primigravid women who subsequently developed hypertension showed an increased sensitivity to Ang II as early as 18 to 22 weeks gestation. Although this was a large series of 192 patients many were Afro-American teenagers and the overall incidence of pregnancy induced hypertension in the study was exceptionally high at 37.5%.

Following these observations several studies have tried to determine whether reduced sensitivity to infused Ang II can be used to predict the subsequent development of PE. The first of these studies to be published was that of Morris and colleagues (1978) who looked at 26 primigravid patients, testing them weekly from 29 to 32 weeks gestation. They found unacceptably high false-positive and false-negative rates and concluded "that assessment of the risk of pregnancy induced hypertension (PIH) with the use of the diastolic pressor response to infused A-II is unreliable" (Morris, O'Grady, Hamilton & Davidson, 1978). Later studies found the test to be more sensitive but still not very specific with false positive rates of 55% (Oney & Kaulhausen, 1982) and up to 42.1% in an Asian population (Nakamura, Ito, Matsui, Yoshimura, Kawasaki & Maeyama, 1986). In 1990 Dekker reported a comparison between the supine pressor test and Ang II infusion at 28 weeks gestation as predictors of the development of PIH in 90 normotensive, nulliparous women. He found that Ang II infusion had a specificity of about 90% and a sensitivity of 92%; the supine pressor test had a similar specificity but a low sensitivity of only 25% (Dekker, Makovitz & Wallenburg, 1990).

All of these studies point to the involvement of the RAS in the pathogenesis of PE and PIH but they are likely to be of limited clinical use because of both the invasiveness of the test and the amount of manpower involved in performing it. Therefore there has been interest in developing a less invasive method of determining an individual's response to Ang II. Platelets are a relatively accessible model of smooth muscle cells; they share many structural and biochemical characteristics and similarities between aspects of platelet behaviour and changes in vascular tone have been described

(Cameron & Ardlie, 1982; Cowley, Stainer, Cockbill & Heptinstall, 1984). A cross-sectional study performed by Baker and colleagues and reported in 1991 found significantly higher platelet Ang II binding in pregnant women who developed PIH compared to women who remained normotensive. Women who also developed proteinuria had even higher (but not significantly so) platelet Ang II binding. Later the same group compared platelet Ang II binding with Ang II infusion in 34 primigravid women, 10 of whom developed PIH. They found that "the use of platelet Ang II binding alone in predicting the outcome of the pregnancies, as assessed using discriminant analysis, was more sucessful than when any of the infusion parameters were used, with 77% of patients being correctly classified" (Baker, Broughton Pipkin & Symonds,1992).

It should be remembered that although there has been great interest in reduced sensitivity to Ang II in pregnancy and loss of this reduced sensitivity in pregnancies which are subsequently complicated by PE the actual mechanism of the reduced sensitivity is unknown. It has been postulated by several groups that it is caused by the elevated levels of PGs found in pregnancy. Following on from this authors have suggested that the comparative increase in sensitivity to ang II in pregnancies which are destined to become pre-eclamptic may be caused by lower levels of vasodilating PGs in these pregnancies and perhaps also higher levels of the vaso-consticting PGs. Work in this field has been hampered by the extremely short half-life of the PGs and by the lack of a suitable animal model for human pregnancy. These theories have been ably summarized by Friedmann, 1988. Following on from these theories of PG imbalance in pre-eclamptic pregnancy, trials of low doses of cyclo-oxygenase inhibitors (eg. aspirin), which reduce thromboxane production whilst prostacyclin production is relatively spared, were designed to see if PE could be prevented. Although the results of early small trials were encouraging the multi-centre collaborative low dose aspirin study in pregnancy failed to support the routine use of prophylactic or therapeutic aspirin (CLASP collaborative group, 1994).

The RAS in the uterus and placenta

Although the components of the RAS were being located in the uteroplacental unit from as early as 1968 (Symonds, Stanley & Skinner, 1968), their significance was not

originally understood. It now appears that the RAS is crucially important in the process of implantation and placentation (Myatt, 1992). Ang II increases vascular permeability which may facilitate endometrial infiltration by fetal trophoblast; it also stimulates angiogenesis. The regulation of vascular resistance and blood flow in the placenta is complex. Although Ang II is a vasoconstrictor it is now known to stimulate the synthesis of prostaglandins E and I (vasodilators) in the uteroplacental unit (Glance, Elder & Myatt, 1985). In low doses Ang II infusion into monkeys has been shown, directly, to increase uterine blood flow and the same effect has been measured indirectly in humans using Doppler velocity ratios (Loquet, Broughton Pipkin, Symonds & Rubin, 1990). In support of this captopril is known to reduce uterine blood flow.

It has been suggested that Ang II is also involved in the regulation of fluid and electrolyte transport across the fetal membranes, as in the renal tubules, but this has not been conclusively shown to occur in human pregnancy.

The uteroplacental RAS may be important in uterine contraction during labour. Ang II is known to cause uterine smooth muscle to contract in vitro and it also increases intracellular iCa^{2+} in human myometrial smooth muscle cells, which in turn causes phosphorylation of myosin light chains and hence contraction. This uterine contraction response to Ang II increases as pregnancy progresses possibly due to loss of progesterone dominance with later gestation. It may be that oxytocin and Ang II act synergistically on the uterus around the time of labour - certainly intracerebral Ang II is known to stimulate pituitary oxytocin release (Broughton Pipkin, 1993).

The fetal RAS

The fetal RAS is not the subject of this thesis. In brief, fetal PRS, assayed in cord blood taken from babies delivered following labour at 35 or more weeks of gestation, is higher than in non-pregnant adults but lower than maternal PRS; this does not apply if the babies have been delivered by elective Caesarean section (Tetlow, 1983). Fetal PRC is higher than maternal levels. No correlation has been demonstrated between maternal and umbilical PRS or PRC suggesting that the maternal and fetal RAS are independent, (Tetlow & Broughton Pipkin, 1983). The use of angiotensin converting

enzyme inhibitors is totally contraindicated in pregnancy even though they are efficient hypotensive agents. This is because of severe adverse effects on the developing fetal renal system causing oligohydramnios, stillbirth and neonatal renal failure (Broughton Pipkin, 1989); this suggests that before birth the RAS plays an important role in fetal cardiovascular and volume homeostasis.

AIMS OF THESIS

Work on this thesis began in 1992 when there was great hope that calcium supplementation given to normal pregnant women might reduce the incidence of pre-eclampsia which caused so much maternal and neonatal mortality and morbidity both nationally and on a global scale. Although one small, single-handed project could not hope to solve such a complex problem it was felt that by concentrating on a sub-group, known to be at increased risk of pre-eclampsia, namely pregnant women with IDDM, useful information could be obtained. As calcium homeostatic mechanisms are known to be linked to the renin-angiotensin system, also implicated in the aetiolgy of pre-eclampsia, and both of these systems are known to be disordered in non-pregnant IDDM it was decided to look at components of both systems. Thus the overall aim was to investigate the hypothesis that pregnant IDDM women have abnormalities in both calcium metabolism and the renin-angiotensin system which might predispose them to the development of pre-eclampsia. To this end several related smaller studies were planned:

1. To conduct a prospective, randomized, double blind, placebo controlled trial on the effect of a daily 1.5g oral calcium supplement, given from mid-second trimester until delivery, on the BP of normal and IDDM pregnant women.

2. To determine and compare fasting concentrations of:

   serum iCa^{2+}, Mg, PO_4, sodium, potassium, creatinine, urate and albumin

   serum iPTH

   plasma renin substrate

   plasma renin concentration

   urinary excretion of the electrolytes, creatinine and albumin

in normal and IDDM pregnant women at 21 and 31 weeks gestation and in the same women when no longer pregnant, looking for both a pregnancy and a disease effect. Also to see if these parameters were affected by the long term calcium supplements being taken by half the women at 31 weeks gestation.

3. To study the short term (4 hours) effect of a 1g oral calcium load, given to normal and IDDM pregnant women at 21 and 31 weeks gestation and to the same women after the end of their pregnancies, on the variables listed above.

4. To look at the diets of the normal and IDDM women during and after pregnancy to see if the calcium intake of the two groups was different.

5. It was also hoped to examine concentrations of 25-DHCC and 1,25-DHCC but this had to be abandoned after promised funds were withdrawn. 1,25-DHCC was measured in a few women before funding was withdrawn but the numbers were insufficient for meaningful analysis.

MATERIALS AND METHODS

LOCATION

The study was carried out at The Royal United Hospital, Bath. The biochemical analyses were performed in the on-site laboratories but hormone analyses were carried out in specialist laboratories as detailed below.

ETHICAL APPROVAL

Local ethical committee approval was sought and obtained for the research protocol and consent form, which was signed by the participating women, as described below.

OUTLINE OF STUDY PROTOCOL

This was a prospective, randomized, double blind, placebo controlled study. A broad outline is given below with each aspect of the protocol then being dealt with in greater detail:

First/early second trimester - recruitment of women

- weight, height and BP measurement

20-22 weeks gestation

24 hour urine collection for Ca, Mg, PO_4, albumin, creatinine and electrolytes

5 hour session in laboratory

Recording of weight and BP.

Baseline fasting blood for elctrolytes, ionized calcium and hormones as described below.

1 hour fasting urine collected and analysed as above

Standard breakfast and 1g oral calcium load followed by hourly blood and urine sampling for analysis as above

Randomization to either 1.5g calcium or placebo daily for the remainder of the pregnancy.

BP recording at fortnightly intervals from this time until delivery.

30-32 weeks gestation - exact repetition of above.

Post-partum, once breast feeding discontinued- exact repetition of above.

RECRUITMENT

Two groups of twenty pregnant women were recruited: the first consisting of normal controls and the second of women with IDDM, the onset of which pre-dated the index pregnancy. All the women were told verbally about the study and asked if they would be interested in participating. If they expressed an interest they were given an information sheet which reiterated the aims of the study and described what would be required of the volunteer. The women were advised to take this home and to discuss it with their partners before coming to a final decision. Following this they signed a consent form which followed guidelines issued by the Bath District Health Authority Research Ethics Committee (see Appendix 1).

Exclusion criteria

Women with a history of renal calculi, essential hypertension, parathyroid disease, or surgery to either the thyroid or parathyroid glands were excluded.

The general practitioners of the participating women were informed of their patient's inclusion in the trial by letter.

Most of the subjects were recruited during the first trimester of pregnancy with a minority entering early in the second trimester (see results). All of the latter group had booked their pregnancy with a midwife before 10 weeks gestation and for these women the booking weight, height and blood pressure recorded by that midwife were used. The remaining majority of women attended the laboratory during the first trimester where they were weighed, their height was recorded and their blood pressure measured.

BLOOD PRESSURE MEASUREMENTS

Throughout the study blood pressure measurements were obtained using a standard mercury syphygmomanometer. Cuff size was determined by upper arm circumference; a standard cuff (bladder size 12 x 22cm) was adequate in almost all cases but for some (arm circumference >33cm) a larger cuff was used (bladder size 14 x 31cm). All subjects were at rest for at least 10 minutes before any recordings were made. Any tight clothing was removed from the non-dominant arm so that there was no

constriction and the cuff could be applied easily. The first readings were obtained with the subjects sitting comfortably in the upright position. The brachial artery was located in front of the elbow and a stethoscope placed directly over it without undue pressure. The cuff was then pumped up rapidly to approximately 30mmHg above the point at which Korotkoff sounds disappeared. Air was released slowly so that the mercury fell steadily at a rate of 2 - 3mm/s. The systolic blood pressure was taken as the point where the first clear tapping sound was heard. The top of the mercury meniscus was read to the nearest 2mmHg. During pregnancy the diastolic blood pressure was taken as the point where the sounds first become muffled (Korotkoff phase IV) as during pregnancy the auscultated Korotkoff sounds may persist to zero due to peripheral vasodilation making the Korotkoff V point (disappearance of Korotkoff sounds) meaningless. If it was present the Korotkoff V point was also recorded. For non-pregnant measurements phase V was used throughout as this corresponds most closely to the true diastolic blood pressure as measured by intra-arterial catheter. Three recordings were taken, the first was discarded and the next two averaged.

Blood pressure was recorded on one occasion during the first trimester between 10 and 12 weeks gestation. It was next recorded at 20 - 22 weeks just before the subject began either the calcium supplements or placebo and from then on was taken at two weekly intervals until delivery. Approximately 85% of the readings were performed by the investigator to eliminate inter-observer error. On occasions when this was not possible the readings were taken by a midwife who had been trained to follow the protocol described above.

ORAL CALCIUM LOADS

These were carried out twice antenatally, as described above, and once postnatally, usually after the subject had stopped breast feeding. As some subjects continued to breast feed for prolonged periods, one for > 2 years, it was not always possible to wait until subjects had completely discontinued feeding; in these cases the postnatal oral calcium load was performed once the frequency of breast feeds had fallen to only one per day. The same protocol was followed for all 3 experiments.

On the day before the study the subjects began a 24 urine collection using a plain bottle. The 24 hour urine samples were later analysed for calcium, magnesium, phosphate, creatinine and microalbumin - see below. Subjects arrived at the laboratory between 08.00 and 09.00hr having had nothing to eat or drink since the evening before Smoking was also not allowed from midnight the night before but as none of the volunteers smoked this was not an issue. The diabetic subjects delayed their normal morning insulin injection until arrival.

On arrival the subjects were asked to empty their bladders and were given 200ml of tap water to drink. Then they were weighed and their height was measured. They then rested in a comfortable chair whilst a 21g cannula (Venflon) was inserted into a suitable vein and 30ml of blood was withdrawn. The cannula was flushed with 5ml of heparinized saline (Hepsal) and secured in position. One hour later they emptied their bladder again using a commode situated in a room adjacent to the laboratory whilst their breakfast was prepared. All the subjects ate a standard breakfast:

2 Weetabix

200ml full fat milk

1 medium slice (35g) wholemeal bread toasted

0.25oz butter

This breakfast provided:

376 calories (1582kJ)

13.8g protein

15.4g fat

48.6g carbohydrate

7.0g fibre

272mg calcium

In addition a 1g calcium load was given as 2 "Cacit" tablets dissolved in 200ml of tap water. These are orange-flavoured effervescent tablets containing 1.25g calcium carbonate which each dissolve to provide 500mg of elemental calcium as calcium citrate. Tap water in the Bath area contains 2.5mg of calcium per 200ml.

The volunteers spent the next 4 hours seated in the laboratory. At hourly intervals they emptied their bladders, drank 200ml of water and had 30ml of blood withdrawn. During this time blood pressure measurements were taken as described above. The subjects were also taken through a dietary questionnaire (Ryan, Kellow, Jphns, Black, Sorenson, Fogelman, 1994) with food portion models to determine their dietary calcium intake (Appendix II). After 4 hours the intravenous cannula was removed and the subjects were given a sandwich lunch after which they were free to go.

PREPARATION AND ANALYSIS OF SAMPLES

The 30ml of blood taken was divided into prepared tubes: 12ml into 2 plain glass tubes which was allowed to clot at room temperature, 12ml into chilled lithium heparin glass tubes, 5ml into a chilled bottle, prepared on site, containing 100µl disodium EDTA, and 1ml into a plain vacutainer - this was also allowed to clot at room temperature. Slightly more than 30ml of blood was removed on the first occasion while the volunteer was still fasting and approximately 3ml were placed in a plain, clotted vacutainer for later measurement of fructosamine The chilled tubes were transferred immediately to a centrifuge and spun at 4°C for 10 minutes at 2,500rpm. The plasma obtained was decanted into pre-labelled small plastic tubes and stored at -20°C within 20 minutes. The clotted samples were spun similarly at room temperature and aliquots of serum were also stored at -20°C.

The volumes of urine passed hourly were measured using a cylinder graded in 2ml divisions and aliquots of approximately 10ml were placed in plastic tubes and stored at -20°C.

The 24 hour urine sample and the vacutainer blood samples were taken to the laboratories of the Royal United Hospital on the day of the study. Fructosamine was measured on the DAX 48 analyser. Essentially the test is based on the ability of ketoamines to reduce nitroblue tetrazolium in alkaline medium to form the dye formazan. The rate of production of formazan is directly proportional to the fructosamine concentration and is measured photometrically at 548nm. The interassay CV varied from 5.67% to 5.98%. Fructosamine, rather than Haemoglobin A1c, was

measured as this gives an insight into glycaemic control over a shorter, and hence, more recent, time period of 2-3 weeks.

Ionized calcium

Ionized calcium was measured on a 1ml clotted blood sample taken into a plain clotted vacutainer, thus excluding air from the sample as exposure to carbon dioxide lowers the pH of the serum sample which raises the ionized calcium value. In addition the serum was analysed as rapidly as possible after transfer to the laboratory. An AVL analyser was used which depends on an ion selective electrode principle.

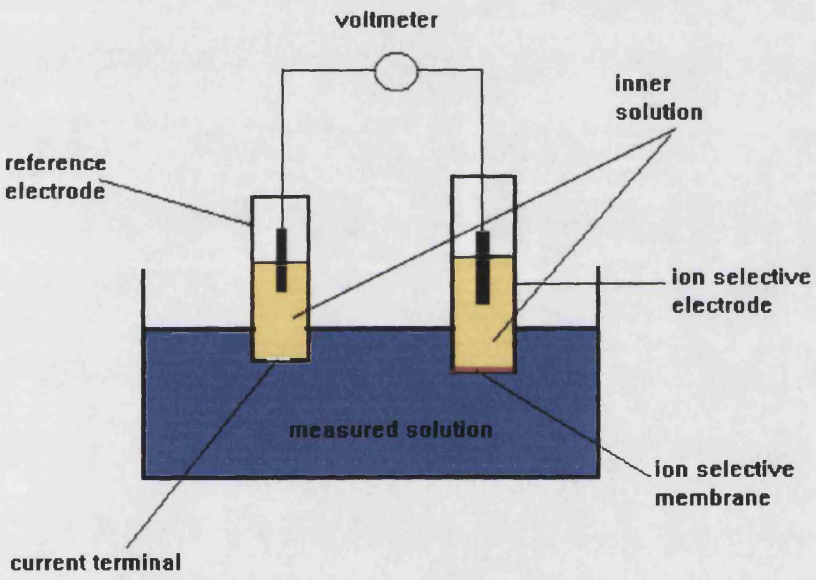


Fig.2.1 Diagram of measuring system for ionized calcium

As shown in Fig. 2.1, an ion (calcium) selective membrane separates the sample whose electrolyte concentration is unknown from an electrode solution whose electrolyte concentration is known. The membrane is constructed so that it undergoes a specific reaction with the electrolyte in the sample and acts as an ion exchanger. Therefore a potential difference is set up across the membrane. The concentration of the inner solution in the electrode is known and this determines the potential on that side of the membrane. On the sample side of the membrane the concentration is unknown but by measuring the potential difference across the membrane with a voltmeter the unknown

ionized calcium concentration can be calculated using the Nernst equation. The interassay CV at 0.8mmol/l $Ca2^{+}$ is 2.5%, improving to 1.3% at 1.5mmol/l.

Measurements of serum and urine electrolytes and glucose

Glucose was measured using the DAX 48 analyser. Glucose is first phosphorylated and then converted to 6-phosphogluconate-δ-lactone by D-glucose-6-phosphate dehydrogenase. The latter reaction concurrently reduces NAD to NADH and this results in an increased light absorbance at 340nm which is directly proportional to the glucose concentration. The interassay CV is 2.9%.

Urine calcium, albumin and creatinine were analysed on the Roche Cobas Bio centrifugal analyser. Urine sodium, potassium, urea, phosphate and magnesium and serum sodium, potassium, urea, creatinine, chloride, urate, phosphate, albumin, magnesium and calcium were analysed on the Johnson & Johnson Ektachem Kodak 750 system. The urine control material was Biorad Lypocheck levels 1 and 2 and the serum control material was Ektachem performance verifier 1 and 2. The interassay CVs were:

Performance verifiers (PERV) 1 and 2 (blood)

	PERV 1 CV(%)	PERV 2 CV(%)
Sodium	0.8	0.7
Potassium	1.7	1.5
Urea	2.0	2.2
Creatinine	1.5	1.1
Chloride	0.8	0.5
Urate	1.4	1.2
Phosphate	1.1	0.7
Albumin	1.6	1.3
Magnesium	2.3	1.4
Calcium	1.6	1.3

Biorad Lypocheck level 1 (urine)

	Sodium	Potassium	Urea	Phosphate	Magnesium
CV(%)	3.6	4.1	3.9	4.9	1.3

Biorad Lypocheck levels 1 and 2 (urine)

	Calcium	Creatinine	Microalbumin
Level 1 CV(%)	3.4	2.1	3.9
Level 2 CV(%)	2.6	2.9	3.3

The assay for urine calcium is a colourimetric method which uses o-cresolphthalein and 8-OH quinolone to eliminate interference from magnesium ions. The reagents are supplied by Biorad.

Serum intact parathyroid hormone

Frozen serum samples were transferred to the Biochemistry Department of the University Hospital of Wales, Cardiff where iPTH was measured using the Ciba Corning "Magic Lite" assay. 200µl of serum, which contains iPTH together with an excess of biologically inert carboxy-terminal fragments, is mixed with antibody labelled with a chemiluminescent acridinium ester which is specific for the amino-terminal sequence of PTH. This binds to the iPTH but not the C-terminal fragments. After an overnight reaction a C-terminal specific monoclonal antibody linked to magnetic particles is added in large excess. This binds both C-terminal fragments and iPTH. The particles are separated on a magnet and washed free of any unbound material. The amount of luminescence associated with the solid phase depends on the amount of hormone "sandwiched" between C-terminal solid phase antibody and acridinium labelled N-terminal antibody and is used to provide a direct measure of the *intact* hormone concentration by comparision with known standard concentrations of synthetic human 1-84 PTH. The limit of detection of the assay is 0.8pmol/l.

Plasma renin substrate and plasma renin concentration

Frozen samples of plasma prepared by mixing blood with disodium-EDTA were taken

to the Department of Obstetrics & Gynaecology at the Queen's Medical Centre, Nottingham for assay. PRC was measured by reacting the renin in samples with excess (heterologous) Aogen under standard conditions. PRS was measured by its ability to generate Ang I from an excess of homologous renin under the same physiological conditions. The inter- and intra-assay CVs for PRS were 12.7% and 6.3% respectively and 10.7% and 5.6% for PRC.

RANDOMIZATION TO ORAL CALCIUM SUPPLEMENTATION OR PLACEBO

After completing their first oral calcium load the subjects were randomly allocated to receive either 1.5mg of elemental calcium in the form of 3 "Calcichew" tablets daily (Shire Pharmaceuticals) or 3 placebo tablets in the form of white lactose tablets supplied by the Pharmacy at Royal United Hospital, Bath. Computerized randomization was carried out separately for the normal and IDDM women to ensure that 10 of each group received calcium and 10 of each group received placebo. Details of the group to which each woman had been assigned were held by pharmacy and only released at the end of the trial. The women themselves were not aware of which they were taking. The pharmacy also kept a record of the number of tablets dispensed to each volunteer. After delivery the women returned their unused tablets to the pharmacy; thus it was possible to calculate how many tablets they had taken and hence their compliance.

STATISTICAL METHODS

Results are expressed as means ± standard error of the mean (SEM) or medians with interquartile ranges (IQR) as appropriate. In some cases the standard deviation (SD) is also quoted to allow comparisons with the work of others. The statistical package for social scientists version 7.5 for Windows was used to analyse data. In general, normally distributed data were compared using the Student's T test (paired or unpaired as appropriate) provided that Fisher's test was not significant. If Fisher's test was significant or if the data were not normally distributed then non-parametric tests were used. Analalysis of variance (ANOVA) with post hoc T test by Scheffe at $p< 0.01$, or Kruskal Wallis analysis of variance with post hoc Mann Whitney U test, for non-parametric data, was used to calculate time-related or pregnancy-related changes in

variables. In keeping with convention $p<0.05$ was regarded as significant and $p<0.01$ highly significant. On occasion, as mentioned in the text, data which were not normally distributed were $\log_{10}$ transformed to create a normal distribution before further analysis was performed.

Wherever scatter graphs are used to illustrate correlations between two variables the equation for the "line of best fit," calculated by linear regression is quoted in the form of $y = a + bx$ where "a" is the intercept on the y axis and "b" is the slope of the line; both "a" and "b" are given with their appropriate SEM. Differences in the slopes and intercepts of regression lines were calculated longhand by T-test. Again in keeping with convention $p<0.05$ was regarded as significant, with $p<0.01$ being highly significant.

Labelling of figures

Normal women are shown in red and IDDM women in green. "Gestation" has been used to describe the different times at which the oral calcium loads were performed including the post-pregnancy study and "time" is used to denote the time from ingesting the breakfast and calcium tablets. Where the data are compared by gestation within one group of women 2nd trimester data are shown in turquoise , 3rd trimester data in pink and the not pregnant data in brown. The phrase "not pregnant" is used in preference to "post-partum" as the women were deliberately not studied until a time when they had recovered physiologically from the pregnancy and any effect of lactation.

RESULTS

3.1A Demographic characteristics of subjects

20 normal pregnant and 20 insulin-dependent pregnant diabetic women were recruited in total. Basic demographic data on the subjects are shown in table 3.1. All were Caucasian.

Table 3.1 Demographic characteristics of subjects

	Normal controls	IDDM	p t test
	n = 20	n = 20	
Maternal age (yrs)	29.9 (0.9)	30.3 (1.1)	ns
Primiparous:Multiparous	9:11	14:6	
Smokers	0	0	
BMI in 1st trimester (kg/m^2)	23.3 (0.7)	24.1 (0.7)	ns
1st trimester systolic blood pressure (mmHg)	112 (2)	113 (2)	ns
1st trimester diastolic blood pressure(mmHg)	73 (2)	72 (2)	ns

Occupations of volunteers

Control women were mainly drawn from hospital staff with 6 doctors, 9 nurses or midwives, 2 receptionists, 1 secretary, 1 laboratory technician and one other who worked in administration at the Ministry of Defence. The IDDM group were more mixed with 5 bank or building society clerks, 4 housewives, 3 shop assistants, 2 civil service workers, 1 doctor, 1 nurse, 1 health care assistant, 1 teacher, 1 personal assistant and 1 in marketing. Diabetic details for this group are shown in table 3.2.

3.1B Details of diabetic control and complications

Table 3.2 Details of the IDDM volunteers

	Age (yrs)	Disease duration (yrs)	1st trimester 24hr insulin dose	1st trimester fructosamine (mmol/l) Normal range 190-285	2nd trimester urine albumin:creatininine ratio (mg/mmol) Normal <2.5	Retinopathy
D1	26	10	56	218	0.3	None
D2	36	23	22	N/A	0.2	None
D3	39	33	56	356	1.4	Treated
D4	37	9	68	232	0.9	Minimal
D5	29	2	18	327	0.8	None
D6	31	11	36	232	1.1	None
D7	26	1	44	273	0.5	None
D8	31	10	60	7.5%*	0.7	Minimal
D9	22	12	16	267	0.9	None
D10	25	18	86	264	0.6	None
D11	26	11	40	247	0.8	None
D12	37	28	24	293	3.9	None
D13	30	22	36	296	1.3	None
D14	30	25	100	305	0.7	Treated
D15	37	10	66	253	0.5	None
D16	31	6	64	281	0.7	None
D17	24	3	48	329	0.5	None
D18	29	19	35	281	0.6	None
D19	33	5	46	5.1%*	0.6	None
D20	27	26	38	222	0.7	Minimal

*These patients were booked in a neighbouring district general hospital and were recruited to increase the number of IDDM women in the study. Haemoglobin A1c was used in this hospital, rather than fructosamine, as an indicator of diabetic control.

The normal ranges are those used outside of pregnancy.

Urinary infection was excluded in all the women at booking.

3.1C Obstetric and perinatal outcome

One normal woman delivered at 30 weeks. The infant went to the Neonatal Intensive Care Unit (NICU) and was discharged well. The others delivered at term (Table 3.3).

Table 3.3 Delivery details for the normal volunteers

	Del. Gestation (wks)	Type of del.	Sex of baby	Wt of baby (kg)	Individ-ualized birth weight ratio (%)	Apgar score at 1 & 5 min	Time spent on Neonatal Intensive care unit (days)
N1	42	NVD	F	4.02	119	6 9	-
N2	38	El LSCS	F	3.69	118	9 9	-
N3	40	NVD	M	3.57	94	9 10	-
N4	39	NVD	M	3.94	111	9 9	-
N5	38	El LSCS	M	3.56	114	10 10	-
N6	41	NVD	F	3.50	97	6 9	-
N7	39	NVD	M	3.57	102	9 10	-
N8	41	NVD	F	4.15	117	9 10	-
N9	40	Forceps	F	3.40	101	9 10	-
N10	40	NVD	F	3.43	96	9 10	-
N11	37	El LSCS	M	3.8	118	9 10	-
N12	39	NVD	M	3.8	116	10 10	-
N13	38	El LSCS	F	2.89	90	9 10	-
N14	41	NVD	F	3.56	97	9 10	-
N15	41	NVD	F	3.54	94	7 7	-
N16	30	Forceps	M	1.68	92(C)	Intubated	56
N17	38	El LSCS	M	3.72	110	9 10	-
N18	40	Em LSCS	M	4.57	122	8 10	-
N19	38	El LSCS	M	3.00	91	9 10	-
N20	38	NVD	M	3.36	98	9 10	-

NVD = normal vaginal delivery; C = centile; El LSCS = elective Caesarean section; Em LSCS = emergency Caesarean section

Individualized birth weight ratios have been given in preference to centiles because these reflect the mother's height, weight and parity and so are a more logical outcome measure of pregnancy than birth weight alone (Wilcox, Johnson, Maynard, Smith & Chilvers, 1993). These are not available for babies at less than 37 weeks or 259 days' gestation and for these babies the centiles have been given and indicated by (C).

The elective LSCS rate for this group of women was 30%, approximately double the Caesarean section rate for the hospital. 5 of the 6 elective operations were repeat procedures where the woman chose not to have a trial of labour. Many of the normal volunteers were doctors and midwives and this group of women are probably more reluctant than the general population to undergo labour in the presence of a uterine scar. They are also less likely to be talked out of a desire for an elective operative delivery by the obstetrician looking after them. The final elective Caesarean section was for a transverse lie.

Details of the deliveries of the IDDM women are summarized in table 3.4. The usual policy for delivery of IDDM women in Bath was to induce labour at 38 weeks. If the woman had had a previous Caesarean section (4 of the volunteers) then an elective repeat Caesarean section was performed rather than allowing a trial of labour. The other elective sections were performed on the two primigravid patients who developed PE, for 2 cases of pre-labour fetal distress (D7 and D8) and at the request of the mother (D15). 5 of the emergency LSCS were performed for failure to progress after induction of labour and the last (D20) was for fetal distress.

In Bath infants of IDDM mothers were routinely admitted to NICU to monitor their blood sugars and this accounts for the 12 NICU admissions of 1-2 days'duration. Longer stays were on account of prematurity - (D3 and D5), while the baby's heart condition was investigated (D13), because of birth trauma (D16) and infant D7, who was delivered because of an abnormal pre-labour cardiotocograph and was born in a poor condition needing intensive resuscitation and ventilation.

Table 3.4 Delivery details for the IDDM volunteers. P = pre-eclampsia

	Del. Gestation (wks)	Type of del.	Sex of baby	Wt of baby (kg)	Individ-ualized birth weight ratio (%)	Apgar score at 1 & 5 min	Time spent on NICU (days)
D1	38	NVD	M	2.44	76	9 10	2^{+}
D2	40*	Em LSCS	F	3.93	112	9 10	1
D3	34 (P)	El LSCS	M	2.06	5(C)	9 10	21
D4	38	El LSCS	M	3.23	85	9 10	2
D5	34 (P)	El LSCS	F	3.23	99(C)	7 9	5
D6	38*	Forceps	M	3.60	110	9 10	1
D7	37	El LSCS	M	2.92	100	Intubated	19
D8	35	El LSCS	F	3.00	90(C)	9 10	-
D9	39*	NVD	M	3.63	109	9 9	1
D10	37 (P)	El LSCS	M	3.12	98	9 10	1^{++}
D11	38*	Em LSCS	M	3.78	114	8 10	2
D12	38	El LSCS	M	3.76	118	8 9	1
D13	37*	Em LSCS	M	4.10	134	9 10	9^{+++}
D14	37	El LSCS	M	4.12	122	8 10	-
D15	38	El LSCS	F	3.75	116	9 10	1
D16	38*	Forceps	F	3.95	130	4 9	8^{++++}
D17	38*	Em LSCS	M	4.59	140	9 10	1
D18	36	Em LSCS	M	3.16	72(C)	9 10	2
D19	38	NVD	M	3.58	107	9 10	-
D20	38*	Em LSCS	M	3.51	112	9 9	1

C = centile

* = induction of labour

NVD = normal vaginal delivery;

El LSCS = elective Caesarean section; Em LSCS = emergency Caesarean section

$^{+}$ congenital hypospadias $^{++}$ undescended testes $^{+++}$ ventricular septal defect

$^{++++}$ delivery complicated by shoulder dystocia - the baby's clavicle was fractured

Women marked by a (P) developed pre-eclampsia (PE), defined as a sustained BP of greater then 140/90mmHg in association with ≥2+ of proteinuria. One of these three women had been randomized to calcium supplementation. The individual case details are summarized below and the results of these women are presented separately.

D3

A 39 year old woman, who had had IDDM for 33 years, was in her second pregnancy. Her first pregnancy had also been complicated by PE. In the index pregnancy her booking BP was 110/70mmHg. She was admitted at 33 weeks with 3+ proteinuria on dipstix testing. Initially her BP was not severely elevated but within a week it rose to 170/95. As the local NICU was full she was transferred to a neighbouring hospital and was delivered of a healthy 2.06kg male infant by elective repeat Caesarean section. Following delivery her BP settled spontaneously to 100/70mmHg and her proteinuria diminished. Notably however when studied 62 weeks after the delivery her 24hr microalbumin result was 65.8mg/l compared to 10mg/l at 20 weeks gestation. This raises the possibility of underlying diabetic nephropathy which may also have been present when the diagnosis of PE was made. This woman was allocated placebo.

D5

This 29 year old primigravida had had IDDM for 2 years. She had no underlying renal disease and booked with a BP of 95/65. She was admitted at 32 weeks gestation with a BP of 160/100mmHg and 2+ proteinuria on dipstix testing. She was monitored closely but at 34 weeks gestation her condition deteriorated and she required intravenous hydralazine to control her BP. She was delivered of a female infant weighing 2.64kg by elective Caesarean section. Following delivery her BP and proteinuria rapidly resolved, confirming a diagnosis of PE. She was unwilling to give blood for a postnatal study but her 24hour microalbuminuria was 5.2mg/l similar to her value of 4.4mg/l at 20 weeks gestation. Her non-pregnant BP was 115/75mmHg.
This women was allocated to calcium supplements.

D10

This 25 year primigravida had had IDDM for 18 years. She also had no underlying nephropathy and booked with a BP of 100/60mmHg. Her pregnancy was uneventful

until 37 weeks when her BP was measured in the antenatal clinic and found to be 155/100mmHg. She had 3+ proteinuria on dipstix testing. She was admitted and delivered a male infant weighing 3.12kg by elective Caesarean section. Subsequently her BP returned to 100/70mmHg and a 24 hour urine collection showed only 26.2 mg/l microalbuminuria. Again PE was felt to be the likely cause of the hypertension in pregnancy. This woman was allocated to placebo.

The results of the women who developed PE are presented separately.

Randomization to calcium supplementation or placebo

This occurred immediately after the women had undergone the 21 week calcium load study. Therefore results for the 31 week study were first analysed in four groups: normal women receiving calcium supplementation; normal women on placebo; IDDM women receiving calcium supplementation; IDDM women on placebo. For nearly all analytes there was no difference between the supplemented and placebo groups but whenever this occurred it is mentioned in the appropriate section of the results chapter. The four subgroups were then re-analysed simply as "normal" or "IDDM" increasing the sample size available for comparison between 21 and 31 weeks' gestation.

3.3D Compliance with prescribed intervention

The number of tablets taken by each individual were determined by subtracting the number returned from the number dispensed by pharmacy. The number that should have been taken was calculated by multiplying the numbers of days between the start of randomization and delivery for each individual patient. Two normal women, on active tablets complained of nausea and only took 21% and 54%. Median compliance in the remaining normal women was 93% (range 82%-100%). None of the IDDM women complained of any side-effects from the tablets. Their compliance was 91%(76%-100%). This was statistically indistinguishable from the normal women.

Although compliance was poor in two of the normal women on active calcium supplements analysis of the results was performed on an "intention to treat" basis.

3.1E Dietary calcium studies

This was assessed by means of a food questionnaire (appendix II) administered by the investigator using appropriate food portion models. Results are shown in table 3.5 and table 3.6 for the women who developed PE.

Table 3.5 Daily calcium intake assessed by questionnaire before calcium supplementation. Mean (SEM) {SD}.

	Pregnant (mid-2nd trimester)		p T-test	Not pregnant		p T-test
	Normal	IDDM		Normal	IDDM	
Ca (mg/day)	1316(80) {310.8}	1574(158) {569.1}	ns	1146(66) {254.3}	1316(139) {502.6}	ns

Although calcium intake was higher in pregnancy in both groups this was of borderline statistical significance with p values of 0.05 and 0.06 for normal and IDDM women respectively.

Table 3.6 Daily dietary calcium of the 3 diabetic women who developed PE

Code	Daily Ca (mg)	
	Pregnant	Not pregnant
D3	1582	1092
D5	2306	1079
D10	1144	1500

Timing of calcium load studies

The study protocol aimed to study all the women firstly at 20-22 weeks gestation, then at 30-32 weeks (one normal volunteer failed to make this appointment because of the death of her mother) and finally postnatally once they had stopped breast feeding. The

last was particularly difficult to achieve as by this stage many of the volunteers had returned to work and were also busy with a new baby making it hard for them to find time to attend hospital for an entire morning. Gestation details for the calcium load studies are shown in table 3.7.

Table 3.7 Gestation when calcium load studies performed. Median (range)

	Normal	**IDDM**	**p**	**Normal**	**IDDM**	**p**	**Normal (time after delivery)**	**IDDM (time after delivery)**	**p**
Wks median range	21 20-23	21 20-23	ns	31 30-35	31 30-35	ns	40 6-75	29 16-54	ns

The women with PE were studied at the following times:

- D3 - 22, 30 and 62 weeks
- D5 - 22, 31 and 84 weeks
- D10 - 21, 34 and 44 weeks

Menstrual details for the after pregnancy study are shown in table 3.8.

Table 3.8 Phase in menstrual cycle when non-pregnant study performed

	Normal women N=15	IDDM women N=15
Follicular phase	2	4
Luteal phase	5	2
Amenorrhoeic	2	2
Combined oral contraceptive pill	5	3
Progesterone only contraceptive pill	1	4

These numbers are too small to allow statistical comparisons to be made.

3.1F Blood pressure results

Control group

Results for the normal women are shown in table 3.9 and for the IDDM women in table 3.10. Although the single first trimester reading was taken at a variety of times between 9 and 12 weeks' gestation they have been grouped together as "12 weeks". The women were not allocated to calcium or placebo until 20 weeks' gestation. However they are shown as subdivided for the first trimester and the not pregnant results; these subgroups were then compared and there was no statistical difference between the baseline blood pressures (BP) of the women allocated to placebo or calcium supplements. As only 3 normal women had BP readings at 40 weeks these are not shown.

Following randomization the women had their BP measurements taken fortnightly. At no gestation was there any difference between either the systolic BP (SBP) or the diastolic BP (DBP) of the women receiving placebo compared with the women receiving calcium supplements. This lack of effect of calcium supplementation on either SBP or DBP in normal women is shown graphically in fig. 3.1 and fig. 3.2.

Table 3.9 BP readings in the normal women. Mean ± SEM

Gestation	SBP	SBP	DBP	DBP
	Placebo	Ca	Placebo	Ca
Not preg	108.4(5.3)	108.6(2.7)	68.0(3.1)	71.6(2.7)
12	112.2(3.9)	113.7(2.1)	72.8(3.4)	70.5(1.8)
20	112.3(4.9)	106.6(5.6)	65.7(8.4)	63.4(3.7)
22	110.3(4.1)	111.6(2.6)	70.1(3.1)	67.1(2.9)
24	109.9(3.1)	110.3(2.2)	66.1(2.7)	60.9(1.8)
26	110.1(1.7)	114.6(2.2)	66.3(3.3)	68.1(2.6)
28	110.9(1.7)	112.3(2.4)	65.8(2.3)	64.4(2.3)
30	111.1(2.2)	113.0(4.3)	66.5(2.3)	65.9(2.8)
32	111.9(3.9)	112.8(4.6)	67.9(2.3)	70.4(3.9)
34	120.8(3.3)	112.2(5.6)	75.2(4.2)	68.7(3.6)
36	118.5(2.3)	110.5(5.2)	74.1(3.1)	69.3(3.2)
38	115.0(2.9)	120.2(4.7)	74.0(3.8)	75.0(2.07)

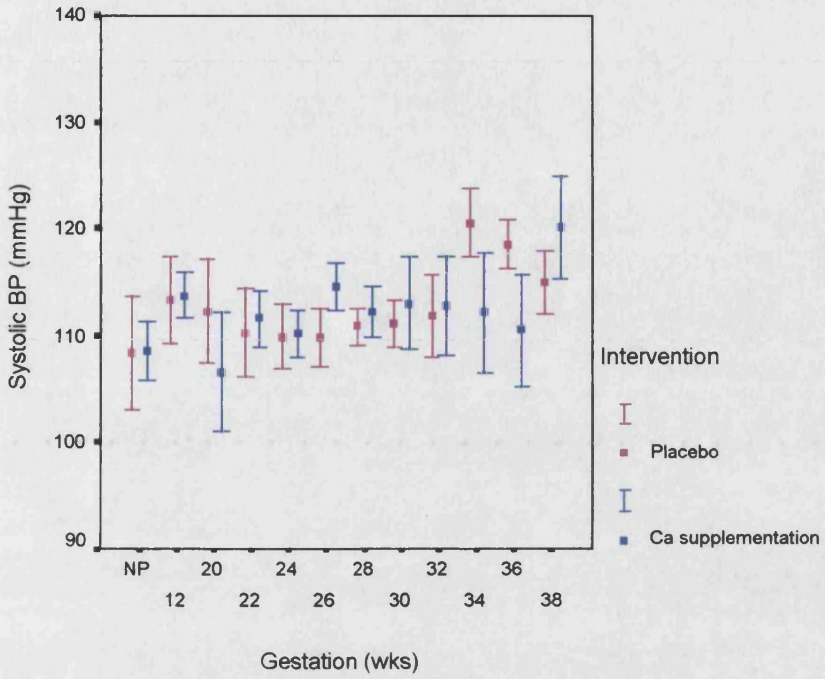


Fig. 3.1 SBP (mean ± SEM) in normal women, with or without calcium supplementation from 20 weeks, plotted against gestation

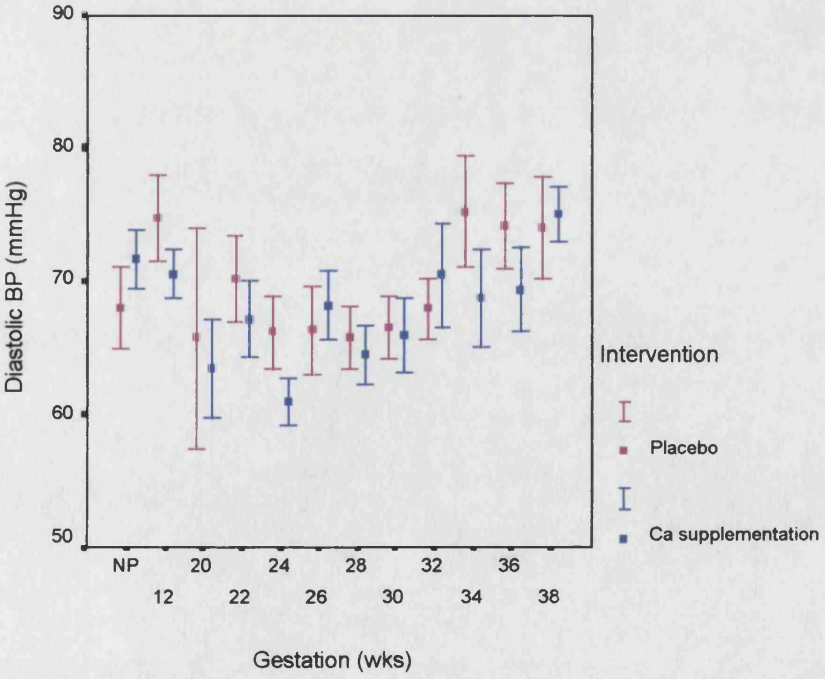


Fig. 3.2 DBP (mean ± SEM) in normal women, with or without calcium supplementation from 20 weeks, plotted against gestation

The data show no relationship between SBP and gestation either as a whole or as placebo/Ca subgroups. However, overall, there was a significant effect of gestation on the DBP readings (ANOVA; $p<0.01$).

IDDM group

Table 3.10 BP readings in IDDM women. Upper results include the women who developed PE whilst these are excluded from the lower results. Mean ± SEM

Gestation	SBP	SBP	DBP	DBP
	Placebo	Ca	Placebo	Ca
Not preg	110.4(3.6)	106.0(3.4)	74.0(2.9)	73.2(3.9)
	113.5(3.8)	105.2(4.0)	73.4(2.2)	74.5(3.4)
12	117.1(2.7)	114.7(4.7)	75.5(3.2)	72.8(3.2)
	118.4(3.2)	115.6(5.6)	74.4(4.2)	73.4(3.9)
20	119.2(1.3)	111.0(5.8)	72.6(2.4)	69.7(6.2)
	119.3(1.7)	111.0(5.8)	71.5(2.8)	69.7(7.2)
22	121.5(4.1)	109.3(4.0)	75.8(2.1)	69.7(2.7)
	123.1(4.3)	108.8(4.9)	76.0(2.4)	69.6(3.3)
24	112.9(3.9)	112.3(5.2)	73.8(2.3)	72.0(4.3)
	113.5(5.2)	109.8(5.5)	74.2(2.9)	70.2(4.8)
26	116.4(5.8)	113.8(4.5)	76.6(3.6)	73.6(3.9)
	122.2(5.9)	110.5(5.5)	78.5(4.5)	71.5(4.2)
28	117.3(3.6)	117.5(5.1)	73.0(2.1)	72.8(4.4)
	120.3(3.9)	114.8(5.3)	72.4(2.5)	74.4(5.0)
30	114.5(1.9)	115.0(7.4)	75.9(1.4)	70.2(4.4)
	115.5(2.4)	111.5(8.4)	75.0(3.7)	70.3(5.7)
32	123.6(4.7)	108.3(7.0)	77.0(2.0)	67.7(5.8)
	120.0(3.7)	107.2(8.5)	76.0(2.0)	66.6(7.0)
34	127.3(7.2)	119.3(12.6)	82.1(3.1)	73.2(4.8)
	121.2(1.5)	109.8(10.1)	78.6(2.1)	70.0(4.4)
36	121.1(1.9)	115.8(4.6)	78.1(3.1)	80.5(4.7)
	121.2(2.3)	115.8(4.6)	76.2(2.9)	80.5(4.7)
38	119.8(5.3)	114	78.3(4.3)	76

n=20 for the upper results until 34 & 36 weeks when n=18, all the women with PE were delivered by 38 weeks; n=17 for the lower results.

Visual inspection of the data suggested that there was no significant difference between the BP readings whether the women who developed PE were included or excluded and this was confirmed by t-test. This shows that the women who went on to develop PE were no different to those who did not develop PE in terms of basal BP before the clinical onset of the disease. Once the women were admitted with a diagnosis of PE no further BP measurements were taken for this thesis.

BP in the IDDM women did not correlate with duration of diabetes, reflecting the fact that women with significant diabetic vascular disease were not recruited to this trial.

Effect of calcium supplementation

Although differences between placebo and supplemented groups were not significant at individual gestational dates, these were significantly different overall being lower with calcium supplementation than placebo both for SBP (ANOVA; $p<0.001$) and DBP (ANOVA; $p<0.05$) as illustrated in figs 3.3 and 3.4.

However when both these figures are examined more closely it is immediately apparent that at 20 weeks when the women were randomized to either calcium or placebo, by chance, the women in the group allocated to calcium had lower SBP and DBP than those who received placebo . To correct for this bias data were recalculated as percentage changes from the 20 week data point and individual t-tests were then performed at each subsequent gestational age. This analysis showed no significant effect of calcium supplementation on either SBP or DBP (fig.3.5 and fig.3.6). Similarly data for normal women recalculated from 20 weeks also showed that calcium supplementation had no effect on percentage change of either SBP or DBP – not illustrated.

Unlike the normal women ANOVA for the IDDM women showed no effect of gestation on either SBP or DBP.

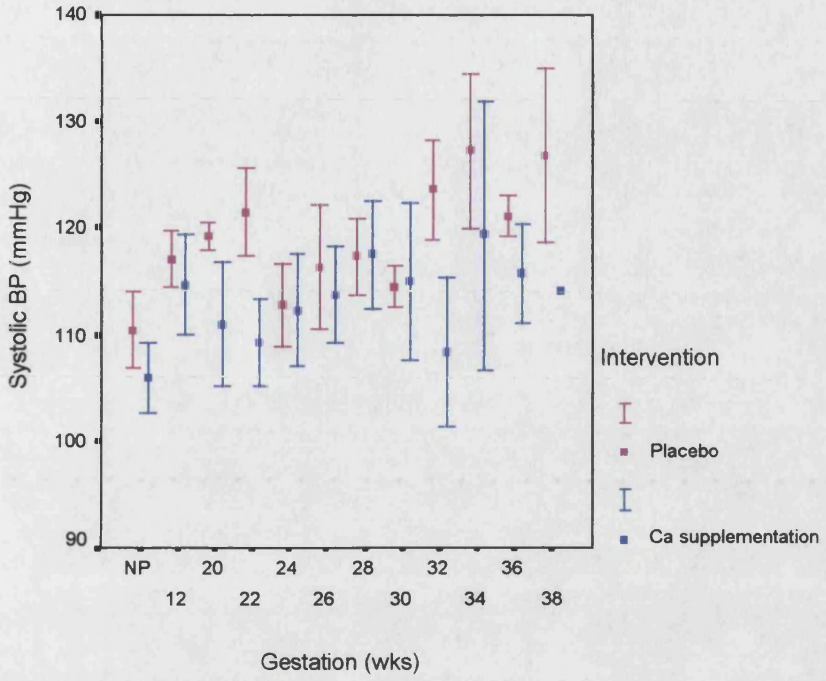


Fig. 3.3 SBP (mean ± SEM) in IDDM women, with or without calcium supplementation from 20 weeks, plotted against gestation. P<0.001, ANOVA.

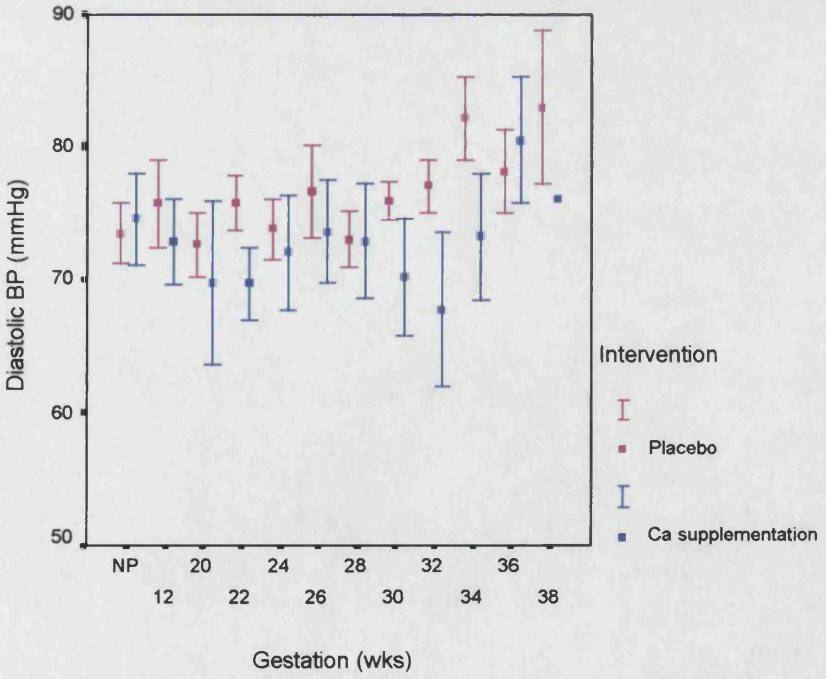


Fig. 3.4 DBP (mean ± SEM) in IDDM women, with or without calcium supplementation from 20 weeks, plotted against gestation . P<0.05, ANOVA.

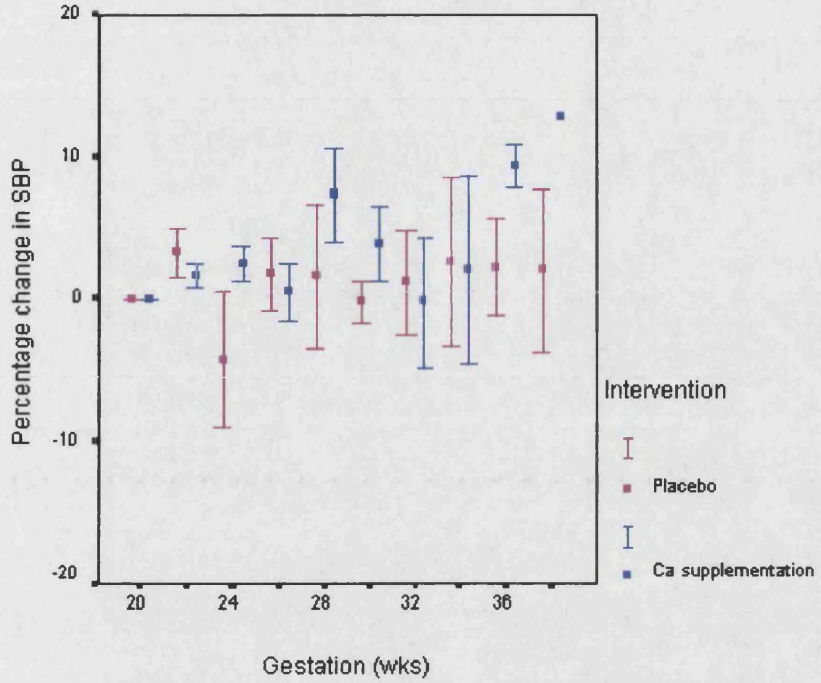


Fig. 3.5 Percentage change in SBP (mean ± SEM) from the SBP at 20 weeks gestation in the IDDM women with or without calcium supplementation plotted against gestation

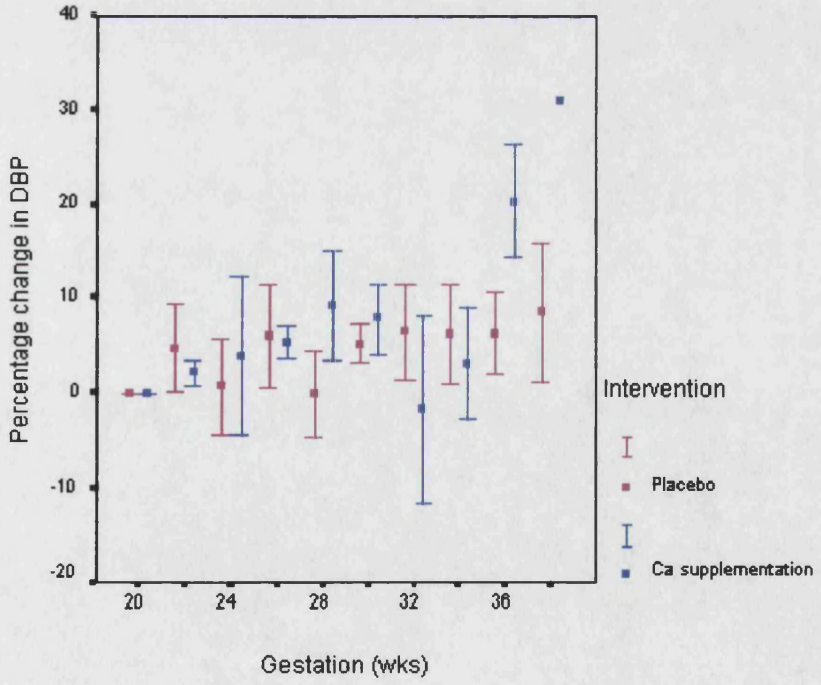


Fig. 3.6 Percentage change in DBP (mean ± SEM) from the SBP at 20 weeks gestation in the IDDM women with or without calcium supplementation plotted against gestation

Differences between the normal and IDDM women

The normal women appeared to have lower BP in general. Simple factorial analysis looking at the effect of gestation on SBP and DBP with diabetic status as the co-variate did show a significant effect with SBP being lower in the normal women – $p=0.01$, and DBP also being lower in the normal group – $p<0.001$.

As mentioned above pregnancy had a significant effect on the DBP in the normal women (ANOVA; $p<0.01$) with values falling after 12 weeks and then rising from 34 weeks until delivery. There was no such difference in the IDDM women. These data for the women on placebo are illustrated in fig. 3.7 while data for the women on calcium supplementation are shown in fig.3 8. It can be seen that there is no "mid-trimester drop" in the IDDM women in either group.

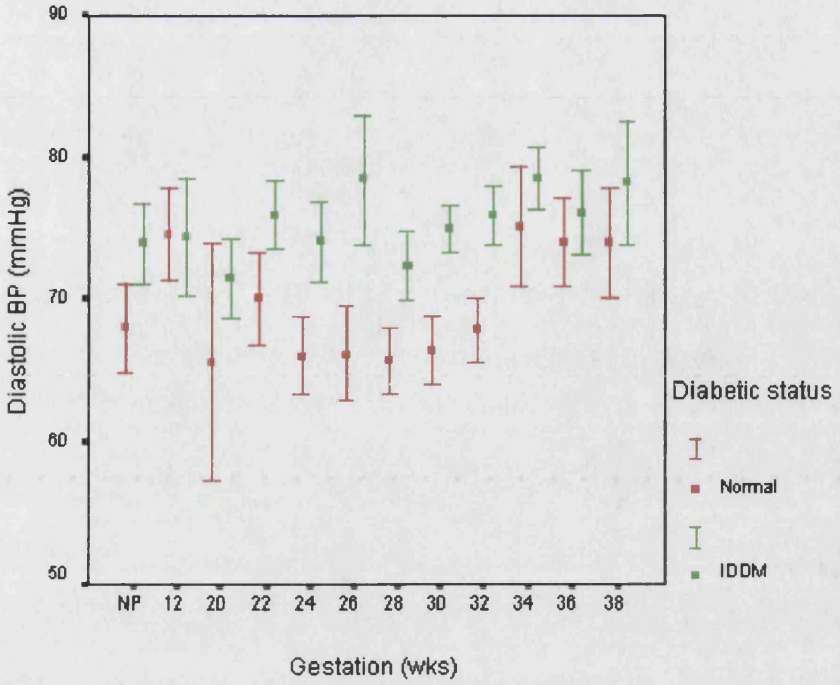


Fig. 3.7 DBP (mean ± SEM) in normal and IDDM women on placebo plotted against gestation

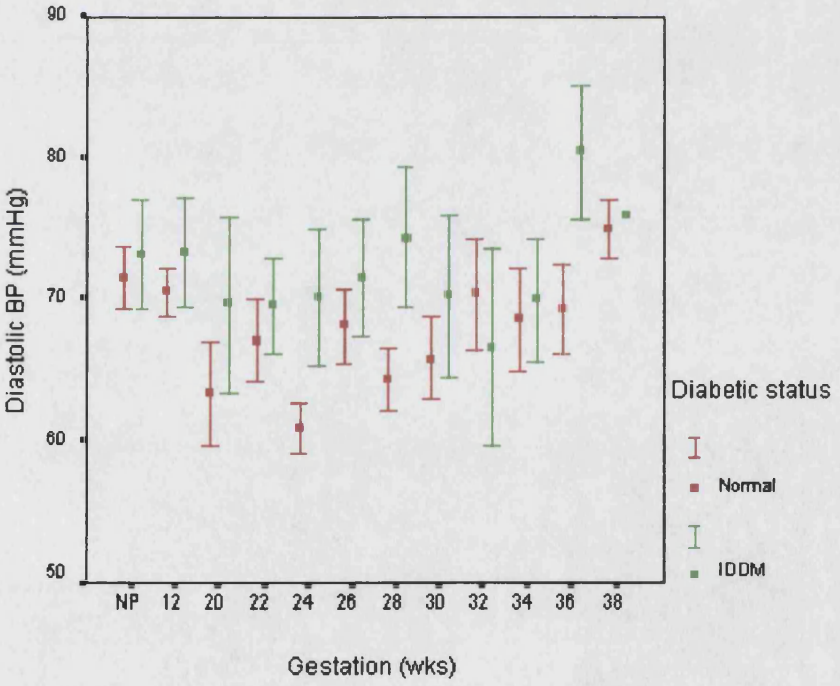


Fig. 3.8 DBP (mean ± SEM) in normal and IDDM women on calcium supplements plotted against gestation

3.2 BASELINE BIOCHEMISTRY RESULTS

In general throughout the results chapters normal women are compared with IDDM women first, looking at the three gestations separately, then changes induced by pregnancy are described in both groups and finally differences in the way pregnancy affects the subgroups are presented. Results for the women who developed pre-eclampsia (PE) are given separately at the end of each sub-section.

3.2A Fasting serum results

1. Normal and diabetic women compared at all three time periods

iCa^{2+}, albumin and electrolytes were compared between controls and the IDDM group at 21 weeks (table 3.11)

Table 3.11 Fasting serum biochemical variables at 21 weeks gestation. Mean ± SEM

	Normal controls n=20	IDDM n=17	P T test
Sodium mmol/l	136.7 (0.7)	137.3 (0.5)	ns
Potassium mmol/l	3.9 (0.1)	4.0 (0.1)	ns
Creatinine μmol/l	54.7 (2.0)	57.5 (2.0)	ns
Ionized calcium mmol/l	1.16 (0.01)	1.18 (0.01)	ns
Magnesium mmol/l	0.72 (0.02)	0.70 (0.02)	ns
Phosphate mmol/l	1.18 (0.02)	1.19 (0.06)	ns
Urate mmol/l	0.22 (0.01)	0.20 (0.01)	ns
Albumin g/l	37.0 (0.5)	36.8 (0.5)	ns

Results for the 31 week study after randomization to either calcium (1.5g daily) or placebo have been subdivided into four groups: normal and IDDM women on placebo and normal and IDDM women on calcium. These are illustrated graphically in fig.3.9. At 21 weeks there were no significant differences in calcium and electrolyte concentrations between the women who were randomized to calcium or placebo. At 31 weeks women receiving calcium had higher serum creatinine levels than women on placebo and lower Mg levels.

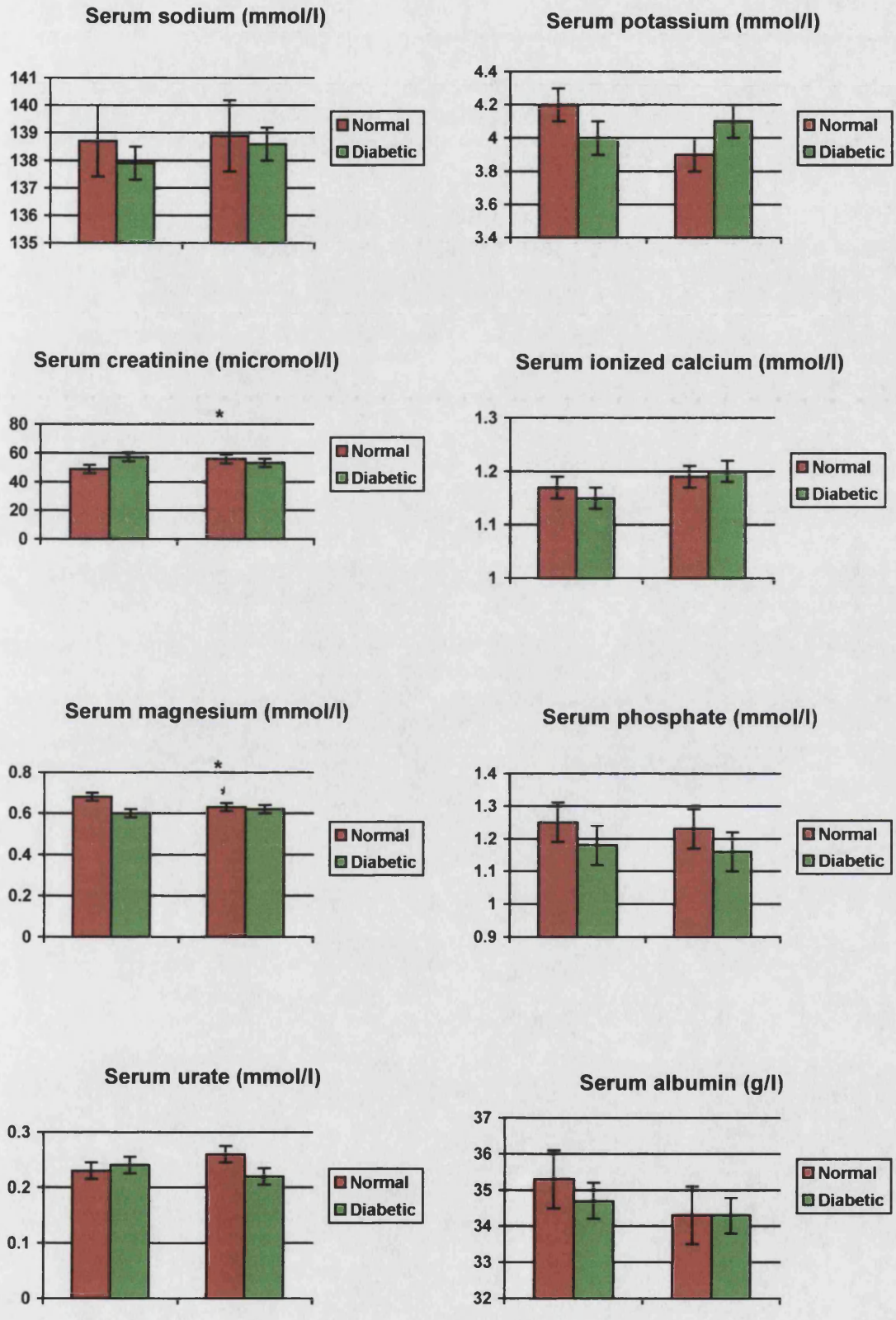


Fig. 3.9 Fasting concentrations of iCa^{2+}, albumin and electrolytes at 31 weeks. Women on placebo are in the left hand columns, those on calcium on the right. Mean ± SEM.
* $p<0.05$ for normal volunteers on placebo compared to calcium supplements

When the women were studied outside pregnanty lower values of serum Mg, PO_4, urate and albumin were found in the IDDM group compared with the normal women. (Table 3.12).

Table 3.12 Fasting serum biochemical values outside pregnancy

	Normal controls N=15	IDDM n=15	P T test
Sodium mmol/l	142.4 (0.5)	140.0 (1.5)	ns
Potassium mmol/l	4.3 (0.1)	4.3 (0.1)	ns
Creatinine μmol/l	62.4 (2.0)	63.7 (2.0)	ns
Ionized calcium mmol/l	1.18 (0.01)	1.20 (0.01)	ns
Magnesium mmol/l	0.79 (0.02)	0.66 (0.02)	< 0.01
Phosphate mmol/l	1.30 (0.04)	1.15 (0.04)	<0.05
Urate mmol/l	0.27 (0.01)	0.21 (0.01)	<0.01
Albumin g/l	46.2 (0.8)	43.0 (1.4)	<0.05

2. The effect of pregnancy on normal women

There was a very highly significant effect of pregnancy ($p<0.001$) on serum sodium, potassium and creatinine which was not affected by calcium supplementation (ANOVA). Post-hoc analysis by Scheffe showed that these three variables all fell significantly by 21 weeks gestation with no further fall between 21 and 31 weeks. Serum ionized calcium was unaffected by either normal pregnancy or by calcium supplementation (fig. 3.10).

Serum PO_4 in the normal women was significantly lower ($p<0.01$) at 21 weeks gestation compared with not pregnant values and from 21 to 31 weeks there was a further slight but not significant rise (fig. 3.11).

Serum Mg levels were also lower at 21 weeks compared to not pregnant values ($p<0.05$). At 31 weeks serum Mg levels were affected by calcium supplementation; at 31 weeks Mg was lower in the supplemented group at 0.63(0.02)mmol/L compared with 0.68(0.02)mmol/L in those on placebo ($p<0.05$) (fig. 3.9). As mentioned above there was no difference between mean serum Mg levels at 21 weeks for these two groups.

Pregnancy, as expected, also had a highly significant ($p<0.01$) effect on serum urate (ANOVA). Post-hoc analysis showed that this was caused by a fall from not pregnant serum urate concentrations to those found at 21 weeks gestation. Values for 21 and 31 weeks were not significantly different although they were slightly higher at 31 weeks, 0.24(0.01)mmol/L compared with 0.22(0.01)mmol/L.

3. The effect of pregnancy on IDDM women

There was no significant fall in serum sodium, PO_4 or urate in IDDM pregnancy and values for the second and third trimesters were the same. Serum potassium and creatinine were both lower in the second trimester compared with the post-partum data but they did not fall further with advancing gestation. Serum Mg was not significantly different between the second trimester and post-partum but it did fall between the second and third trimesters from 0.70(0.02)mmol/L to 0.60(0.02)mmol/L ($p<0.01$). Calcium supplementation status had no effect on serum magnesium levels which were identical at 0.60(0.02)mmol/L in the two subgroups (fig.3.12). Serum iCa^{2+} remained the same at all three gestations, regardless of supplementation status (fig. 3.10).

4. Comparison of the effect of pregnancy on normal and IDDM women

Pregnancy in normal women was associated with a fall in serum albumin and all the electrolytes measured except for iCa^{2+}. In contrast in IDDM pregnancy there was no fall in serum sodium. Serum potassium and creatinine were less at 21 weeks than post-partum ($p<0.01$ and $p<0.05$ respectively) but did not fall between the second and third trimesters. iCa^{2+} behaved similarly in both groups of women remaining stable at all gestations (fig. 3.10).

Serum PO_4 and Mg were lower ($p<0.05$ and $p<0.01$) in diabetic women outside pregnancy compared to the control group. As both fell significantly with pregnancy in the normal women, but not in the diabetic women, at 21 weeks gestation there was no difference in either fasting PO_4 or Mg (figs.3.11 & 3.12). PO_4 appeared to rise slightly between the second and third trimesters in the normal women but this difference was not significant.

There was no difference between the normal and diabetic groups in third trimester fasting PO_4 either when the women were looked at in the subgroups of placebo / supplemented (fig. 3.11) or when these groups were combined (not shown).

In both groups of women Mg levels fell between the second and third trimesters when the supplemented and placebo groups were considered together. However when the women were subdivided it became clear that, as mentioned above, calcium supplementation resulted in lower serum Mg levels in the normal women; this difference was not apparent in the IDDM women (fig. 3.12).

Serum urate behaved in a similar way in that it was lower in the IDDM women outside pregnancy but at 21 weeks there had been a fall in the normal women and not the diabetic women so second trimester values were the same in the two groups (fig. 3.9). There was a non-significant rise between 21 and 31 weeks in the normal women; in contrast serum urate did increase significantly ($p<0.05$) from 21 to 31 weeks in the IDDM women.

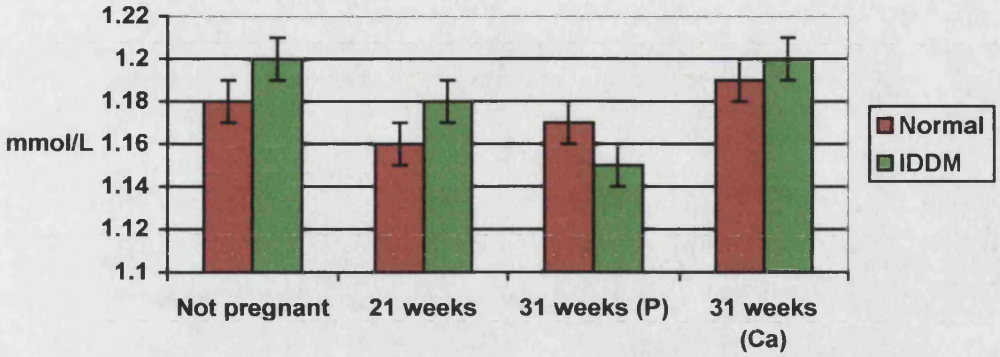


Fig 3.10 Fasting serum ionized calcium – mean ± SEM. P(placebo);Ca(supplemented)

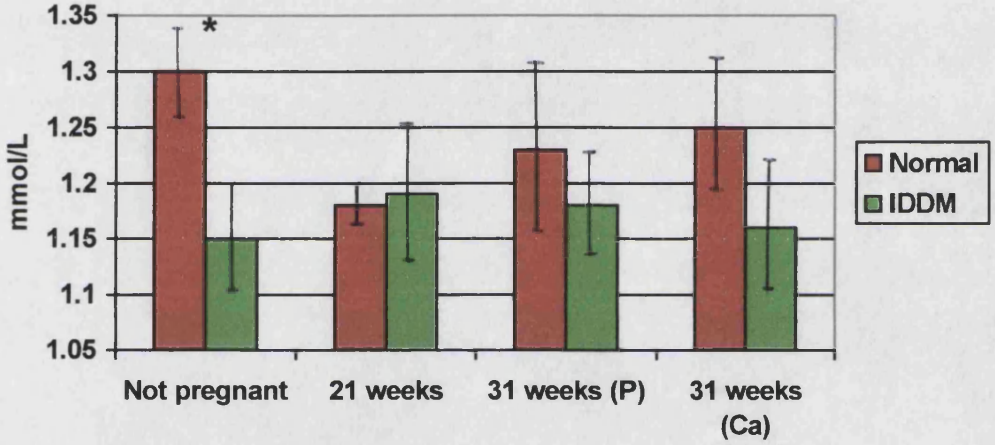


Fig.3.11 Fasting serum phosphate – mean ± SEM . P(placebo);Ca(supplemented)
***p<0.05 comparing non-pregnant normal and IDDM women**

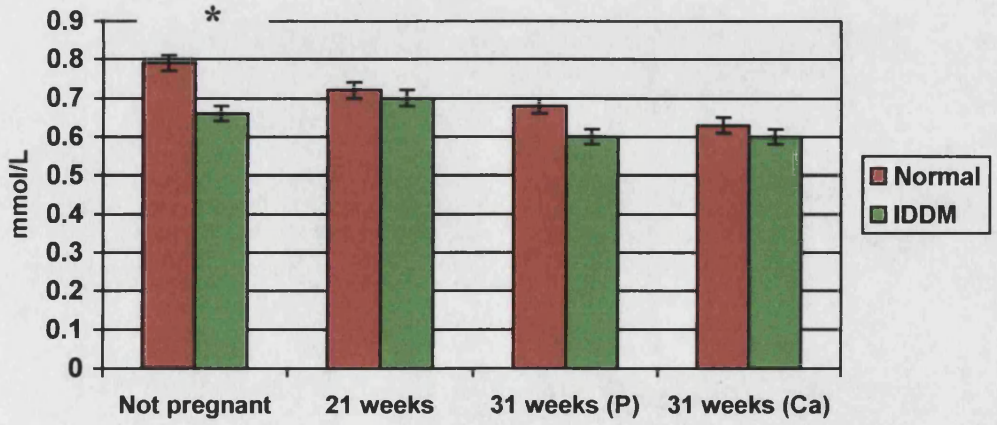


Fig.3.12 Fasting serum magnesium – mean ± SEM. P(placebo);Ca(supplemented)
***p<0.01 comparing non-pregnant normal and IDDM women**

5. Diabetic women who developed PE

D5 was unwilling to give blood when not pregnant so serum electrolyte and albumin results outside of pregnancy are only available for D3 and D10. It can be seen (Table 3.13) that during pregnancy the women who subsequently developed PE tended to have higher basal fasting levels of sodium, potassium, creatinine, Mg and PO_4; iCa^{2+} results appeared similar. D3 had an unusually high albumin result at 21 weeks but this had returned to the normal range by 31 weeks. D10 had raised urate levels at both 21 and 31 weeks despite not developing clinically apparent PE until 37 weeks.

Table 3.13 Fasting values for D3, D5 and D10 at all time periods

	D3			D5			D10		
	NP	**21**	**31**	**NP**	**21**	**31**	**NP**	**21**	**31**
Sodium mmol/l	139	139	138	N/A	138	138	135	138	141
Potassium mmol/l	4.5	4.4	4.4	N/A	4.6	4.5	4.3	4.1	4.5
Creatinine μmol/l	67	95	79	N/A	79	64	62	80	89
Ionized calcium mmol/l	1.15	1.17	1.17	N/A	1.19	1.21	1.20	1.21	1.16
Mg mmol/l	0.67	1.00	0.69	N/A	0.9	0.7	0.58	0.80	0.84
Phosphate mmol/l	1.29	2.33	1.07	N/A	1.45	1.17	1.21	1.25	1.12
Urate mmol/l	0.13	0.16	0.20	N/A	0.18	0.22	0.23	0.33	0.59
Albumin g/l	42	46	33	N/A	38	32	44	36	31

FASTING PLASMA GLUCOSE RESULTS

Predictably these were always significantly higher in the IDDM women (Table 3.14)

Table 3.14 Fasting plasma glucose concentration (mmol/L). Mean (SEM)

	21 weeks	31 weeks	Not pregnant
Normal	3.8(0.1)	3.7(0.1)	4.2(0.1)
IDDM	6.6(0.8)*	5.8(0.7)*	10.7(1.1)*

*$p<0.01$ comparing normals with IDDM

In the normal women pregnancy had a significant ($p<0.05$) effect on fasting plasma glucose concentration; in the IDDM women it had an even greater effect with $p<0.001$ (ANOVA for both). Post hoc testing by Scheffe showed no difference between the 21 and 31 week data but with both being significantly different ($p<0.01$) from the non-pregnant data. The dramatic reduction in fasting plasma glucose in the IDDM women indicates how motivated these women were to achieve tight diabetic control throughout their pregnancy.

PARITY

Parity did not affect fasting levels of serum sodium, potassium, creatinine, iCa, Mg, PO_4, urate or albumin, or fasting plasma glucose in either the normal or the IDDM women but it should be remembered that the numbers in the multiparous group of IDDM women were very small (n=6).

3.2B Creatinine clearance

Creatinine clearances were calculated from both the 24 hour urine collections and for the basal fasting hour of the experiment, and when these were compared they were not statistically significantly different from each other. One of the IDDM patients (D7), despite repeated instructions failed to collect all urine passed in 24 hours on two occasions. These two collections were therefore known not to be accurate and were excluded from analysis. One hour values in this patient were 117.1ml/min and 113.9ml/min respectively. Table 3.15 summarizes the creatinine clearance results calculated form the 24 hour urine collections. Creatinine clearances for the IDDM women have been calculated both including and excluding results for the women who went on to develop PE. It can be seen from table 3.16 that the individual creatinine clearances in D3 and D10 were lower than the average values for the IDDM women.

At 21 weeks the creatinine clearances of the women who went on to receive calcium and placebo were compared and were statistically the same. At 31 weeks both normal and IDDM women on calcium supplements had similar creatinine clearance results compared with the women on placebo (Table 3.15).

Table 3.15 Creatinine clearance calculated from 24hr urine collections (ml/min). Mean ± SEM.

	21 weeks	31 weeks combined	31 weeks (placebo)	31 weeks (Ca)	Not pregnant
Normal	135.6(5.7)*	146.6(7.4)!	148.6(13.4)	144.7(7.3)	111.3(14.7)
IDDM + PE	112.1(5.5)	117.5(8.1)	125.1(13.3)	108.2(7.4)	102.8(7.7)
IDDM – PE	118.0(4.9)	121.7(8.8)	123.7(14.0)	108.1(8.4)	109.7(7.4)

*$p<0.05$ comparing normal with IDDM including and excluding PE
!$p<0.05$ comparing normal with IDDM including PE

Pregnancy had a significant effect on creatinine clearance in the normal women with significant increases from the not pregnant values to 21 weeks and a further rise by 31 weeks. Creatinine clearances also increased during pregnancy in the IDDM women although in this case the significant rise occurred between the not pregnant values and the 21 week data with no further rise with increasing gestation (Wilcoxon signed ranks). This pattern was seen whether or not the women who subsequently developed PE were included or excluded from the analysis although the rise from non-pregnant

to 21 week values was more significant if the women who developed PE were excluded (p=0.008 vs p=0.03). From table 3.16 it can be seen that pregnancy had a minimal effect on the creatinine clearances of the women who went on to develop PE.

Table 3.16 Creatinine clearance (ml/min) for women who developed PE

	21 weeks	31 weeks	Not pregnant
D3	91.6	116.3	103.3
D5	113.9	108.1	109.7
D10	99.5	89.6	97.7

3.2C 24 hour urinary calcium, Mg and PO_4 excretion

Table 3.17 shows the urinary excretion per 24 hours of calcium, Mg and PO_4. Surprisingly whether or not the women were taking calcium supplements did not affect the 24 hour excretion of any of these ions. Therefore when the 31 week data were analysed further they were treated as a unit rather than being subdivided.

Table 3.17 24 hour urinary excretion rates (mmol). Median (IQR).

		21 weeks	31 weeks		Not pregnant
			Placebo	Ca	
Ca	Normal	7.5(6.4-9.7)	8.5(4.6-14.3)	10.1(8.8-12.4)	2.8(2.2-5.5)
	IDDM	6.4(3.8-9.8)	6.1(5.0-10.5)	7.5(5.6-11.8)	2.4(1.7-8.1)
Mg	Normal	4.8(4.3-6.8)	4.5(3.6-6.8)	5.9(5.0-6.4)	4.0(1.9-4.9)
	IDDM	5.5(4.8-6.4)	5.4(5.0-6.9)	6.8(3.8-7.0)	5.5(4.0-7.4)
PO_4	Normal	28.8(21.5-32.2)	23.4(19.4-23.4)	21.7(17.9-28.1)	24.2(18.7-26.1)
	IDDM	25.6(21.5-42.6)	26.2(18.4-35.3)	21.1(15.8-37.2)	23.5(20.2-32.1)

There was no difference in 24 hour calcium excretion between the normal and the IDDM women at any of the three times when the women were studied. Gestation had

a significant effect (Kruskall Wallis ANOVA) on 24 hour urinary calcium excretion in both the normal women ($p<0.001$) and the IDDM women ($p<0.05$) (fig. 3.13).

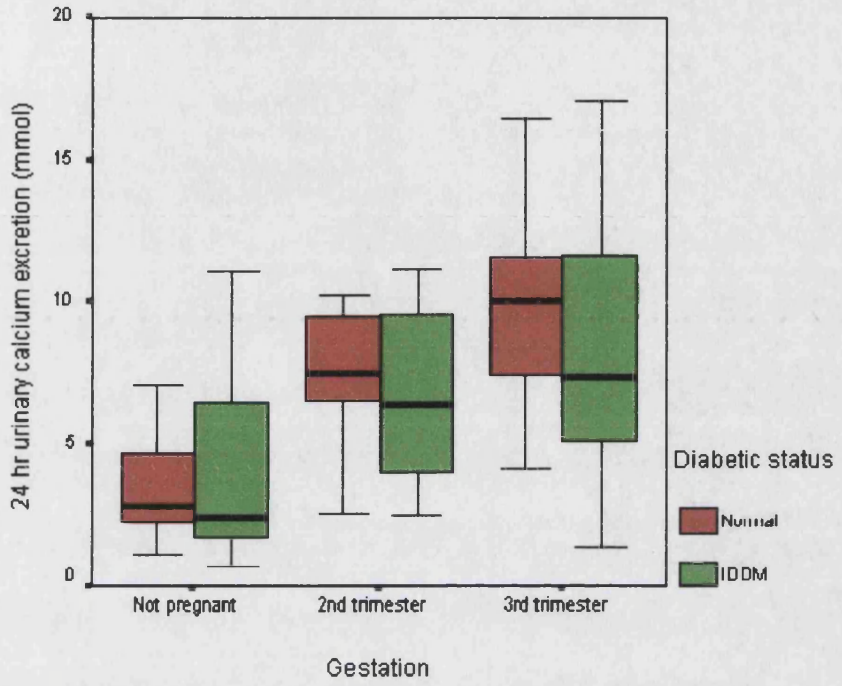


Fig. 3.13 24 hour urinary calcium excretion rates in normal and IDDM women during and after pregnancy. Values shown are medians, interquartile range and absolute range.

Table 3.18 shows urinary calcium excretion rates in the women who developed PE. D10 was significantly hypocalciuric both during and after her pregnancy.

Table 3.18 24hr urinary excretion of calcium in women who developed PE (mmol)

	21 weeks	31 weeks	Not pregnant
D3	7.6	7.9	8.7
D5	16.2	12.4	7.3
D10	2.4	0.6	0.6

24 hour excretion of Mg was the same in normal and IDDM women at all three times. Analysis of the data by Kruskall Wallis ANOVA showed no effect of gestation in

either group of women (fig.3.14) although the trend was for Mg excretion to increase in normal women.

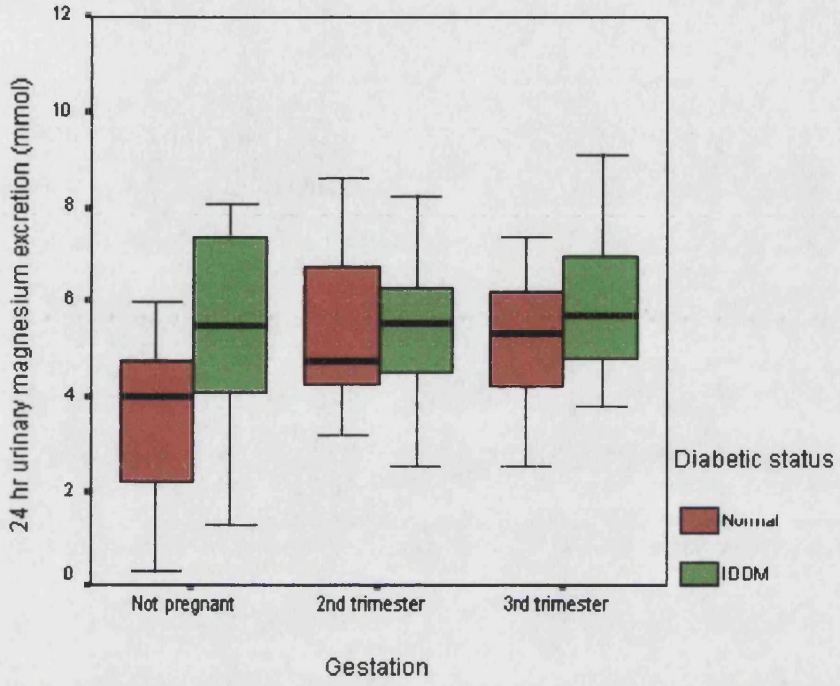


Fig. 3.14 24 hour urinary Mg excretion rates in normal and IDDM women during and after pregnancy. Values shown are medians, interquartile range and absolute range.

Data for the women who became pre-eclamptic are shown below in Table 3.19. These were not different from the remainder of the IDDM women's results.

Table 3.19 24hr urinary excretion of Mg in women who developed PE (mmol)

	21 weeks	31 weeks	Not pregnant
D3	4.7	7.7	5.9
D5	7.8	6.4	3.6
D10	4.9	3.5	5.7

24 hour excretion of phosphate was also the same in the two groups of women and there was no demonstrable effect of gestation (Fig. 3.15).

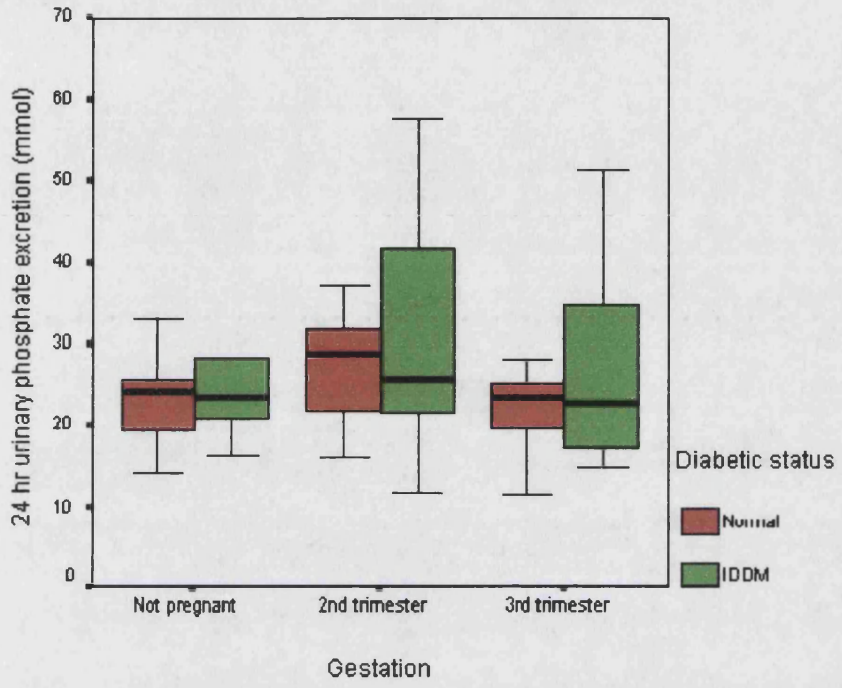


Fig. 3.15 24 hour urinary PO_4 excretion rates in normal and IDDM women during and after pregnancy. Values shown are medians, interquartile range and absolute range.

Data for the women who became pre-eclamptic are shown below in table 3.20. As with calcium (Table 3.18), but not Mg (Table 3.19), D10 had low urine excretion rates.

Table 3.20 24 hr urinary excretion of PO_4 in women who developed PE (mmol)

	21 weeks	31 weeks	Not pregnant
D3	22.4	29.2	24.6
D5	47.6	35.4	15.9
D10	16.3	12.3	11.2

3.2D Baseline fasting fractional excretion (FE) of specific cations

FE rates which take account of individual renal function (see below) were also determined and analysed since the normal and IDDM groups had different baseline renal function. These were calculated using data from the first hour of the experiment ie. the hour before the patients ate their breakfast and the calcium load. The formula used was:

$$\text{Fractional excretion X(\%)} = \frac{[\text{Urinary X mmol/L}] \times [\text{Plasma creatinine mmol/L}] \times 100}{[\text{Urinary creatinine mmol/L}] \times [\text{Plasma X mmol/L}]}$$

where X = ion

There were no significant differences between FE rates of sodium, potassium, calcium, Mg or PO_4 when the women were subdivided into those receiving placebo and calcium (p values ranged from 0.3 to 0.9), therefore the data from 31 weeks were grouped together to obtain two larger groups rather than four smaller groups.

In normal women pregnancy did not have a significant effect on FE of sodium, potassium, Ca, Mg or PO_4 (ANOVA) - Table 3.21 and Fig.3.16. When FE rates between 21 and 31 weeks were compared only the FE of PO_4 was different being higher at 21 weeks (p=0.01).

Table 3.21 Fractional excretion rates in normal women (%). Mean ± SEM.

	21 weeks n=20	31 weeks n=18	Not pregnant n=15
FE sodium	0.66(0.07)	0.55(0.06)	0.59(0.07)
FE potassium	13.55(1.00)	10.40(1.24)	13.55(1.43)
FE Ca	1.34(0.19)	2.02(0.41)	1.09(0.27)
FE Mg	2.19(0.38)	2.26(0.41)	1.92(0.36)
FE phosphate	8.83(0.83)	6.22(0.5)*	8.08(1.65)

*p=0.01 comparing 21 and 31 weeks

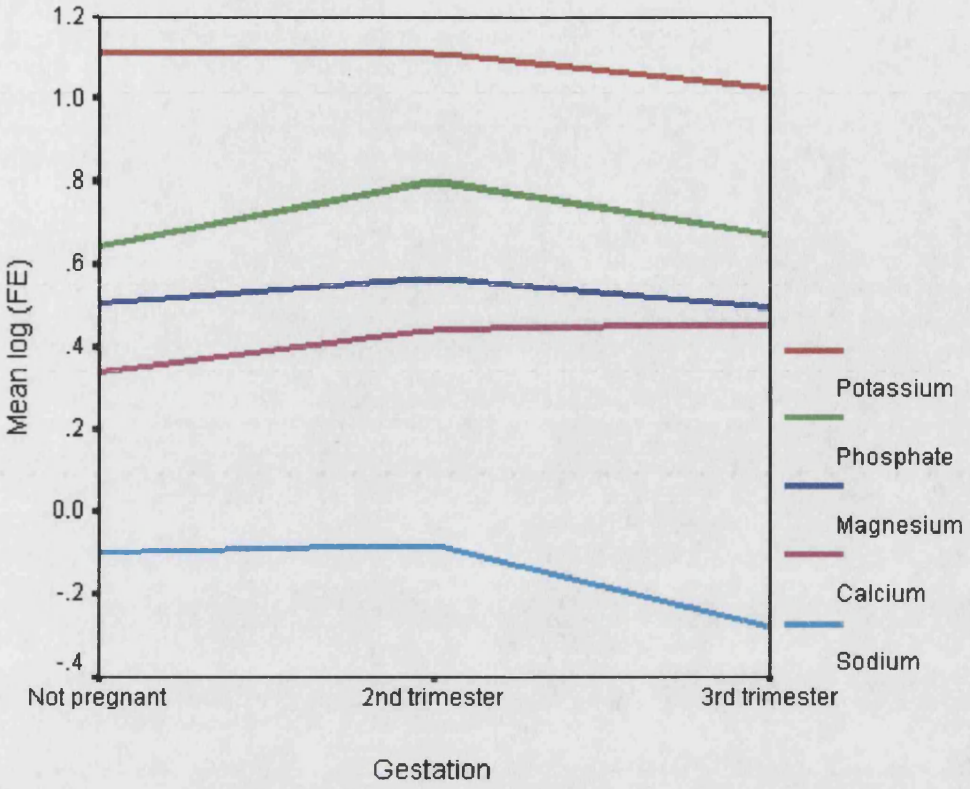


Fig. 3.16 Fractional excretion (FE) rates of varying cations and PO_4 at different gestations in normal volunteers.

In the IDDM women the FE of sodium increased in pregnancy comparing both 21 and 31 weeks with the non-pregnant value ($p<0.05$ for both), (Table 3.22 and fig.3.17). FE sodium was unchanged between 21 and 31 weeks. ANOVA confirmed a significant effect of gestation ($p<0.05$). FE of potassium, Mg and PO_4 were unaltered by pregnancy. The FE of calcium was higher at 21 weeks than outside pregnancy ($p<0.05$); this difference was not apparent at 31 weeks which was indistinguishable from 21 weeks and non-pregnant (Table 3.22 and fig. 3.17).

Table 3.22 Fractional excretion rates in IDDM women (%)

	21 weeks n=17	31 weeks n=17	Not pregnant n=13
FE sodium	0.89(0.09)*	0.78(0.07)*	0.54(0.08)
FE potassium	13.59(1.49)	11.25(1.26)	10.72(1.64)
FE Ca	1.77(0.28)!	1.54(0.29)	0.96(0.13)
FE Mg	2.60(0.35)	2.32(0.32)	2.93(0.50)
FE phosphate	12.30(1.00)	11.47(1.33)	11.24(1.63)

*$p<0.05$ and !$p<0.01$ comparing pregnant and not pregnant

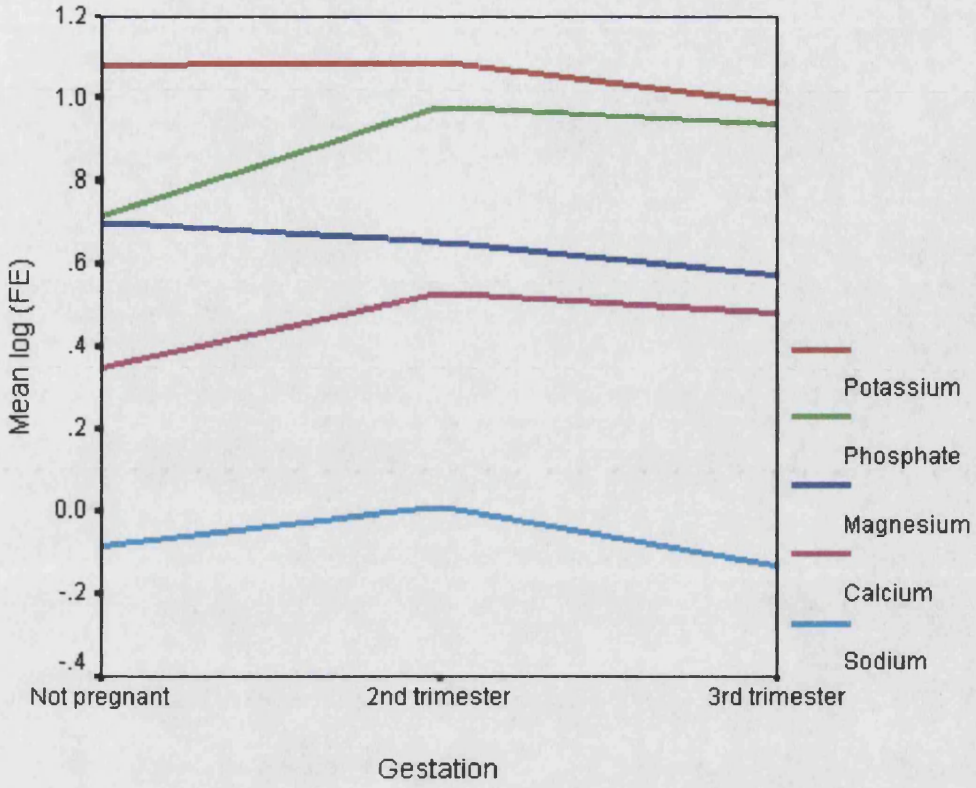


Fig. 3.17 Fractional excretion (FE) of varying cations and PO_4 at different gestations in IDDM volunteers.

FE rates in normal and IDDM women compared

When the normal and IDDM women were compared FE of potassium and calcium were not affected by disease status (2 way ANOVA). Differences in FEMg between the normal and IDDM women just failed to reach significance outside pregnancy ($p=0.07$) but were the same at 21 and 31 weeks. Outside pregnancy FE of sodium and PO_4 were also the same in the two groups. However within pregnancy FE of sodium and PO_4 were higher in the diabetic women. At 21 weeks the higher FE of sodium in the IDDM group was almost significant at $p=0.05$; by 31 weeks this difference was apparent ($p=0.01$). At 21 weeks FE of PO_4 was higher in the IDDM women ($p<0.05$), by 31 weeks this was even more significant ($p<0.01$) (fig.3.18).

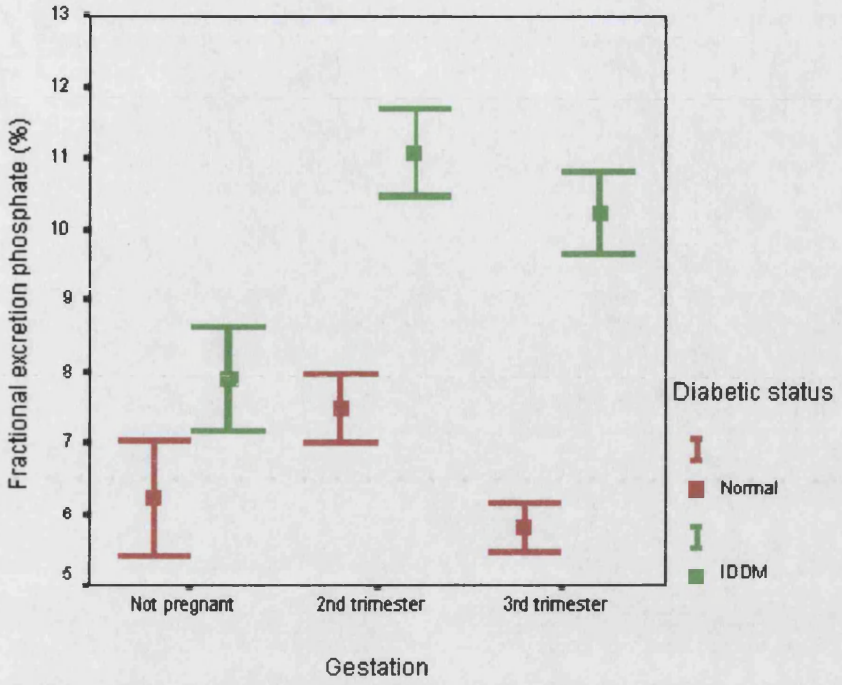


Fig 3.18 Fractional excretion of phosphate in normal and IDDM women, mean ± SEM.

FE rates of the IDDM women who developed PE are shown in Table 3.23. The FE of potassium at 21 weeks was high in D5 and D10 and the FE of calcium and Mg were high in D3 and D5 at all gestations.

Table 3.23 Fractional excretion rates (%) in women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
FE sodium	0.95	0.65	0.35	0.73	0.61	1.44	0.43	0.74
FE potassium	11.95	8.89	4.36	25.76	10.14	26.42	4.12	8.59
FE Ca	5.90	2.60	3.18	4.12	4.34	0.66	0.34	0.22
FE Mg	5.43	4.28	4.48	5.09	3.68	0.63	2.87	3.87
FE phosphate	14.20	14.69	13.61	22.28	16.54	8.88	8.96	12.86

PARITY

Parity had no significant effect on creatinine clearance in either group of women. It also did not effect baseline FE rates of sodium, potassium, calcium, Mg or PO_4 in either the normal or the IDDM women.

3.3 CALCIUM LOAD EXPERIMENTS

3.3A Serum electrolytes

i. Sodium and potassium

Fasting values of serum sodium in the normal and IDDM women have been presented in section 3.2A. Serum sodium remained the same for the four hours following the standard breakfast and calcium load at all gestations in both groups of women. (Table 3.24). Calcium supplement status had no effect on the serum sodium response to the calcium load in either group of women. In general the IDDM volunteers tended to have lower levels of serum sodium than the normal women and this reached statistical significance as indicated in Table 3.24. Small numbers, particularly in the sub-groups at 31 weeks, may account for the failure to reach significance at other points.

Table 3.24 Serum sodium (mmol/l) in normal and IDDM volunteers. Mean ± SEM.

Time from meal		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
Fasting	Normal	136.7(0.7)	138.8(1.3)	138.9(0.5)	142.4(0.5)
	IDDM	137.3(0.5)	137.9(0.8)	138.6(0.4)	140.0(1.5)
1 hour	Normal	138.0(0.4)	140.0(0.5)	139.0(0.6)	143.1(1.0)
	IDDM	136.2(0.5)*	137.6(0.3)	138.1(0.4)	136.9(3.1)*
2 hour	Normal	137.8(0.4)	139.3(0.3)	138.9(0.4)	143.1(0.6)
	IDDM	136.5(0.5)	136.2(1.1)	137.4(0.3)	140.1(1.2)
3 hour	Normal	137.3(0.4)	136.6(2.5)	138.4(0.4)	142.1(0.8)
	IDDM	136.6(0.4)	136.4(1.2)	137.4(0.3)	137.4(2.4)*
4 hour	Normal	136.6(0.7)	138.3(0.6)	138.7(0.5)	142.0(0.7)
	IDDM	136.1(0.5)	137.6(0.7)	137.5(0.3)	140.0(0.9)

*$p<0.05$ comparing normal and IDDM

Pregnancy, as shown in section 3.2A, was associated with a significant ($p<0.01$) fall in serum potassium in both groups of women. Like serum sodium, potassium

concentrations were unaffected by the breakfast and calcium load (ANOVA performed on data for each gestation), see Table 3.25. calcium supplement status had no effect on the response of serum potassium to the meal at 32 weeks gestation.

Table 3.25 Serum potassium (mmol/l) in normal and IDDM volunteers. Mean ± SEM

Time from meal		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
Fasting	Normal	3.9(0.1)	4.2(0.1)	3.9(0.1)	4.3(0.1)
	IDDM	4.0(0.1)	4.0(0.1)	4.1(0.1)	4.3(0.1)
1 hour	Normal	3.7(0.1)!	3.9(0.1)	3.7(0.1)	4.2(0.1)
	IDDM	4.0(0.1)	4.0(0.1)	3.9(0.1)	4.2(0.1)
2 hour	Normal	3.8(0.1)	4.0(0.1)	3.9(0.1)	4.3(0.1)
	IDDM	4.0(0.1)	4.0(0.1)	4.0(0.1)	4.3(0.1)
3 hour	Normal	3.9(0.1)	3.9(0.1)	4.1(0.1)	4.3(0.1)
	IDDM	4.0(0.1)	4.0(0.1)	4.0(0.1)	4.1(0.1)
4 hour	Normal	3.8(0.1)	4.0(0.1)	4.1(0.1)	4.2(0.1)
	IDDM	3.9(0.1)	3.8(0.1)	3.8(0.1)	4.1(0.1)

!$p<0.01$ comparing normal with IDDM and normal with the fasting result in the normal women

When the data were analysed comparing individual hours there was a significant fall ($p<0.01$) between the fasting and 1 hour results in the normal women at 21 weeks; this was the only time and the only sub-group of women when such a difference was apparent and may be the result of a type I error associated with performing multiple comparisons. The low 1 hour result in the normal women at 21 weeks is likely to be the cause of the significant difference between this figure and the result for the IDDM women.

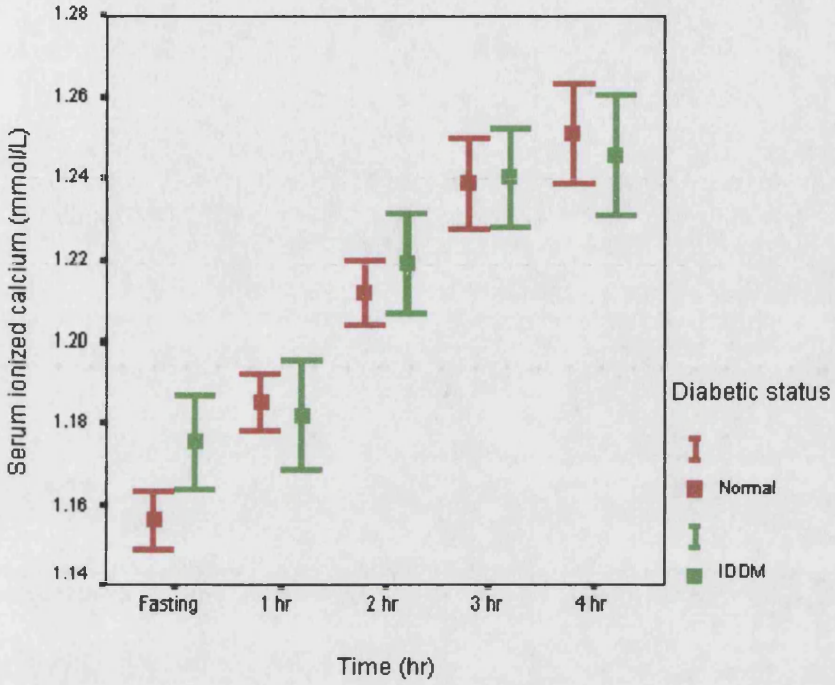
ii Serum iCa^{2+}

Serum iCa^{2+} rose very significantly ($p<0.01$ one-way ANOVA) in both normal and IDDM women at all three time periods studied following the oral calcium load as shown in Table 3.26 and fig. 3.19. Post-hoc testing showed a significant difference between fasting and 4 hr values outside pregnancy in both groups of women while at 21 and 31 weeks there was a significant increase by 2hr in the normal women and by 3 hours in the IDDM women. Serum iCa^{2+} was continuing to rise when the studies ended 4 hours after the calcium load at all three gestations.

Table 3.26 Serum iCa^{2+} (mmol/l) in normal and IDDM volunteers. Mean ± SEM.

Time from meal		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
Fasting	Normal	1.16(0.01)	1.17(0.10)	1.19(0.02)	1.18(0.01)
	IDDM	1.18(0.01)	1.15(0.01)	1.20(0.02)	1.19(0.01)
1 hour	Normal	1.18(0.01)	1.19(0.02)	1.19(0.02)	1.19(0.01)
	IDDM	1.18(0.01)	1.17(0.01)	1.20(0.02)	1.20(0.01)
2 hour	Normal	1.21(0.01)	1.21(0.02)	1.22(0.02)	1.21(0.01)
	IDDM	1.22(0.01)	1.18(0.02)	1.22(0.03)	1.22(0.02)
3 hour	Normal	1.24(0.01)	1.23(0.03)	1.25(0.03)	1.22(0.01)
	IDDM	1.24(0.01)	1.23(0.01)	1.25(0.03)	1.25(0.02)
4 hour	Normal	1.25(0.01)	1.24(0.02)	1.27(0.03)	1.24(0.02)
	IDDM	1.25(0.01)	1.22(0.01)	1.26(0.02)	1.27(0.02)

When the 31 week data were subdivided according to whether the women were receiving calcium supplements or placebo there was no difference in fasting serum iCa^{2+} between the two subgroups of normal women. There was also no statistical difference between the fasting values for the two subgroups of IDDM women although on visual inspection of the data it appeared that those women on placebo had lower fasting levels of serum iCa^{2+} (Fig. 3.20).



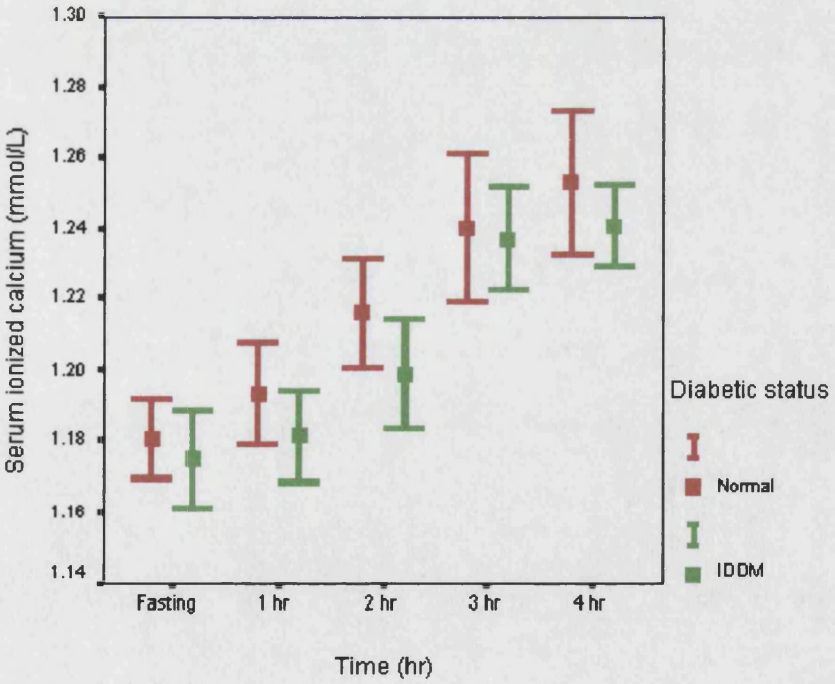


Fig. 3.19 Serum iCa2+ concentrations in normal and IDDM women following oral calcium loading at (upper graph) 21 weeks gestation, (lower graph) 31 weeks gestation and (over page) in the non-pregnant women. Mean ± SEM.

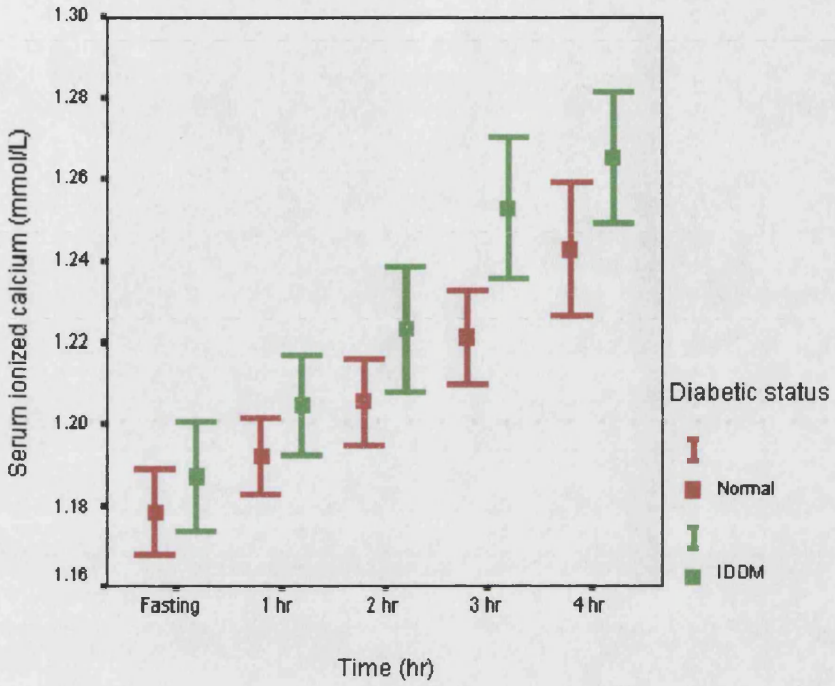


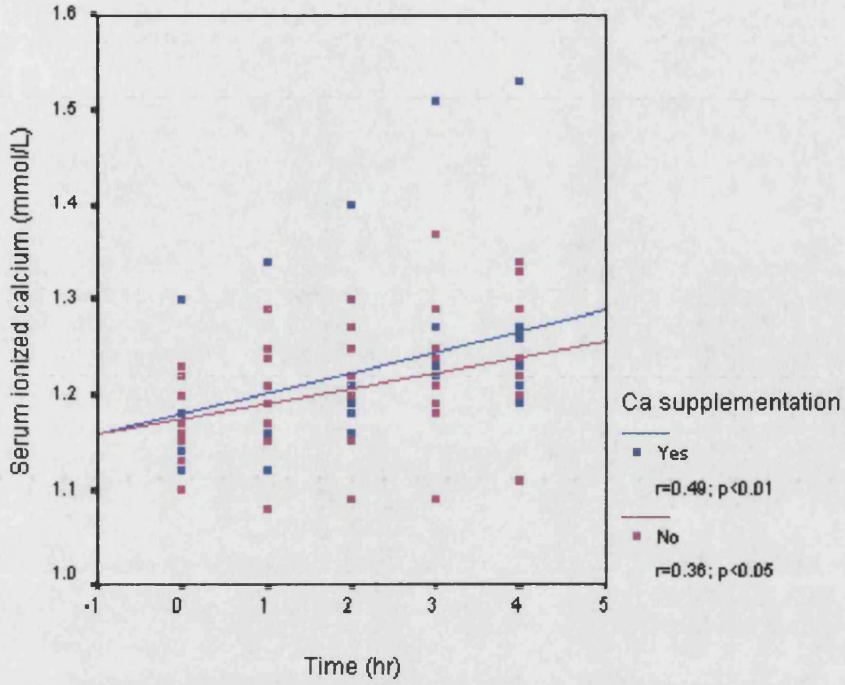
Fig. 3.19 (cont) Serum iCa2+ concentrations in non-pregnant normal and IDDM women following oral calcium loading. Mean ± SEM.

Fig. 3.20 shows the increase in serum iCa^{2+} with time comparing the women on placebo with those on supplements; when the slopes of the graphs were compared (t-test) there was no difference between the rate of rise of serum iCa^{2+} with time between those in the supplemented subgroup compared with those on placebo in either the normal or the IDDM women.

As mentioned in the Methods section the equations for the scatter graphs illustrating correlations are given in the form of

$$y = a + bx$$

where "y" is the variable on the y-axis; "a" is the intercept on the y-axis; "b" is the gradient of the slope.



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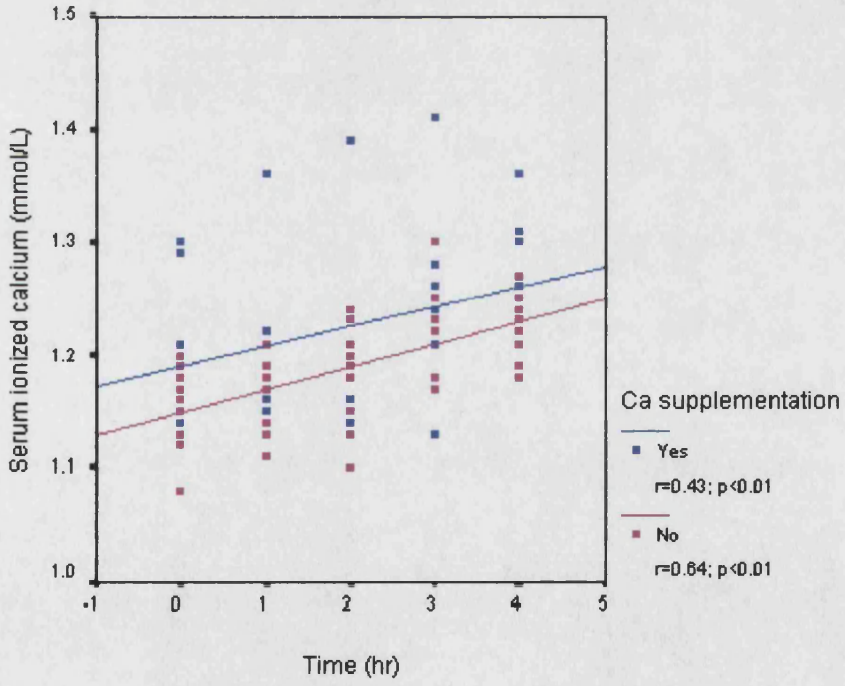
Placebo iCa2+ = 1.18(0.02) + 0.04(0.01)[time]



Ca iCa2+ = 1.19(0.02) + 0.05(0.01)[time]
Placebo iCa2+ = 1.15(0.01) + 0.05(0.01)[time]

Fig. 3.20 The relationship between serum iCa^{2+} and time following the oral calcium load in normal (upper graph) and IDDM (lower graph) women subdivided according to the calcium supplement status of the women

iii Serum magnesiun

Serum Mg levels (Table 3.27) were not affected by the oral calcium load. The repeated samples after the standard meal confirmed that the IDDM women had lower serum Mg levels than the control group outside pregnancy ($p<0.01$). This difference was not apparent during pregnancy presumably because of the slight fall ($p<0.05$) in serum Mg which had occurred in the control women by the second trimester and which persisted into the third trimester.

Table 3.27 Serum magnesium (mmol/l) in normal and IDDM volunteers. Mean ± SEM.

Time from meal		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
Fasting	Normal	0.72(0.02)	0.68(0.02)	0.63(0.02)$	0.78(0.02)!
	IDDM	0.70(0.02)	0.60(0.02)	0.60(0.02)	0.66(0.01)
1 hour	Normal	0.72(0.02)	0.67(0.02)	0.60(0.02)$	0.81(0.02)!
	IDDM	0.68(0.03)	0.59(0.02)	0.60(0.02)	0.63(0.03)
2 hour	Normal	0.70(0.02)	0.67(0.02)	0.63(0.02)	0.80(0.02)!
	IDDM	0.70(0.03)	0.60(0.02)	0.60(0.02)	0.67(0.01)
3 hour	Normal	0.71(0.02)	0.68(0.01)	0.66(0.02)	0.77(0.03)!
	IDDM	0.71(0.03)	0.61(0.02)	0.61(0.02)	0.66(0.02)
4 hour	Normal	0.73(0.01)	0.68(0.02)	0.67(0.02)	0.77(0.04)*
	IDDM	0.69(0.03)	0.62(0.02)	0.62(0.02)	0.67(0.01)

$p<0.05$ and !$p<0.01$ comparing normal and IDDM women

$ $p<0.05$ comparing supplemented and placebo groups of normal women

iv Serum phosphate

In the normal women serum PO_4, unlike serum Mg, was affected by the oral calcium load. In normal women in the second trimester serum phosphate fell 1 hour after the calcium load and levels remained low at hour 2. Following this they rose again and by 4 hours were indistinguishable from fasting values. These changes were very significant (ANOVA, $p<0.001$). Post-hoc testing by Scheffe confirmed that the differences occurred between the 1 and 2 hour values and the 4 hour value. At 31 weeks there were no differences in fasting serum PO_4 levels in normal women regardless of whether they were on calcium supplements or not; after the oral calcium load both groups followed the 21 week gestation pattern with an initial fall and a return to baseline by 4 hours. Again this was significant (ANOVA, $p<0.001$). These changes are shown graphically in fig. 3.21. A similar pattern occurred in the non-pregnant woman but just failed to reach significance with $p=0.05$.

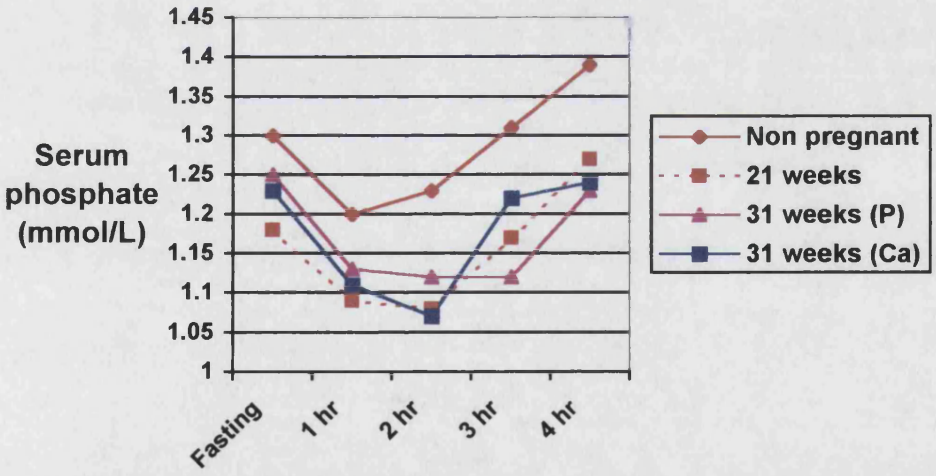


Fig. 3.21 Mean serum phosphate in normal women subdivided by gestation and by the calcium supplement status of the women

The IDDM women behaved similarly to the normal women, in that their serum PO_4 fell initially and then rose back to basal levels in response to oral calcium loading in both trimesters of pregnancy. However these changes were not significant in the IDDM women unlike the normal women (ANOVA). Again the supplement status of the women had no effect on their serum PO_4 response. It was mentioned above (section

3.2A) that fasting serum PO_4 was significantly ($p<0.05$) lower in non-pregnant IDDM women than controls. Despite starting from a lower baseline the non-pregnant IDDM women appeared to show initial suppression after oral calcium, followed by a rise in serum PO_4 back to baseline values. However the difference between the fasting and 1 hour values just failed to reach significance $p=0.05$ and similarly to the non-pregnant normal women the effect of the calcium load was not significant (ANOVA) (fig. 3.22).

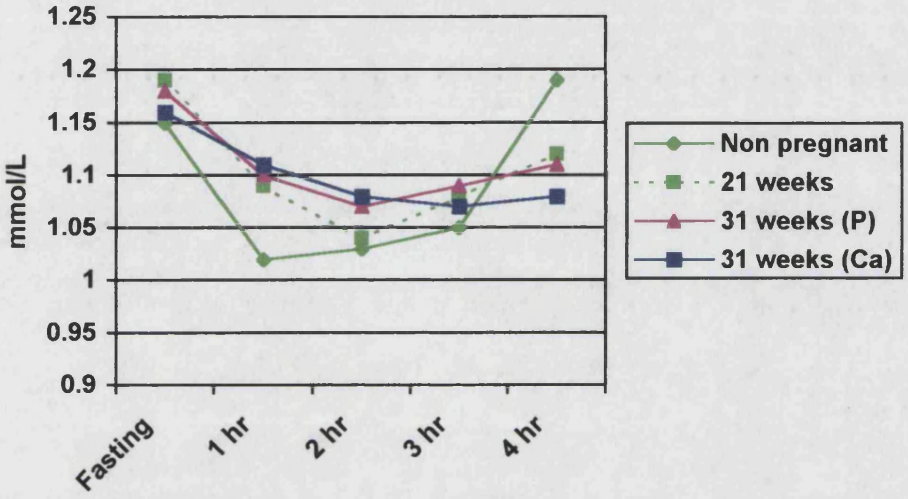


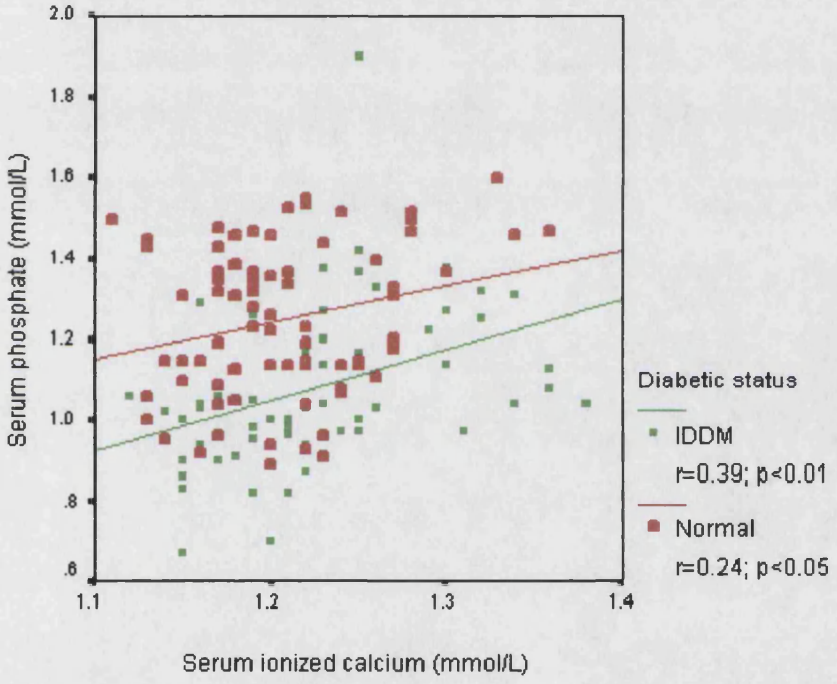
Fig. 3.22 Mean serum phosphate in IDDM women subdivided by gestation and by the calcium supplement status of the women

v Serum urate and albumin

Concentrations of these substances were not affected by oral calcium loading in either group of women on any of the three occasions studied. Data not shown.

3.3B Correlations between serum ionized calcium, phosphate and magnesium

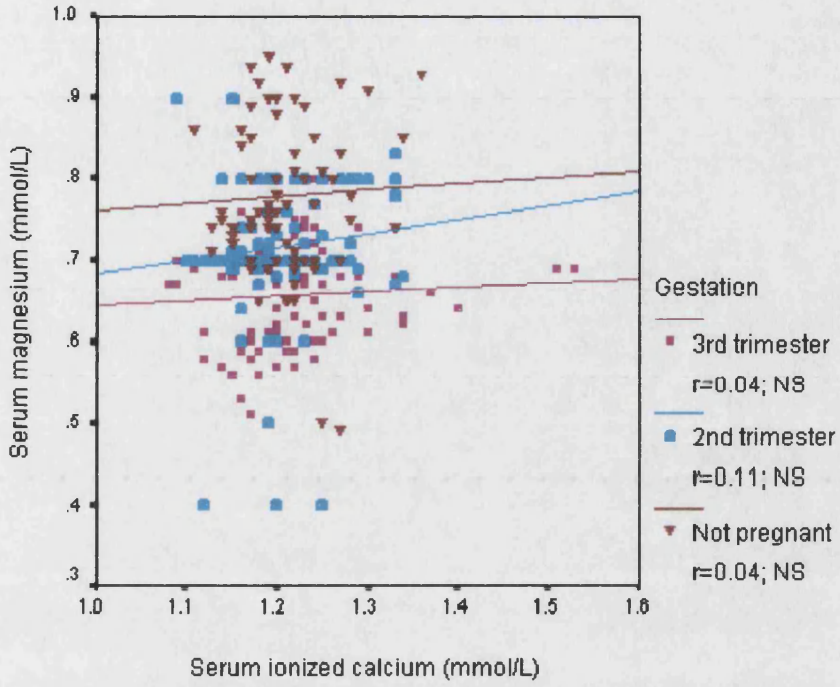
Serum iCa^{2+} showed a positive correlation with serum PO_4 in normal women at 21 weeks gestation ($p<0.001$) and in the non-pregnant state ($r=0.24$; $p<0.05$); there was no correlation at 31 weeks ($p=0.7$). In the IDDM women no correlation was apparent at 21 weeks but at 31 weeks there was a positive correlation ($p=0.016$) and again this was apparent in the non-pregnant diabetic women ($r=0.39$; $p<0.01$) (fig.3.23). When the y-axis intercepts were compared by T-test they were significantly ($p<0.01$) different.



Normal $[PO_4] = 0.18(0.52) + 0.89(0.43)[iCa^{2+}]$
IDDM $[PO_4] = -0.44(0.46) + 1.24(0.37)[iCa^{2+}]$

Fig. 3.23 Correlation between the concentration of serum PO_4 and serum iCa^{2+} in non-pregnant normal and IDDM women

In normal women there was no correlation between serum iCa^{2+} and serum Mg at any gestation (fig.3.24). However in the IDDM women (fig. 3.24) there was a significant positive correlation both at 21 weeks ($r=0.28$, $p<0.01$) and outside pregnancy ($r=0.33$, $p<0.01$). At 31 weeks there was no correlation ($r=0.08$, NS).



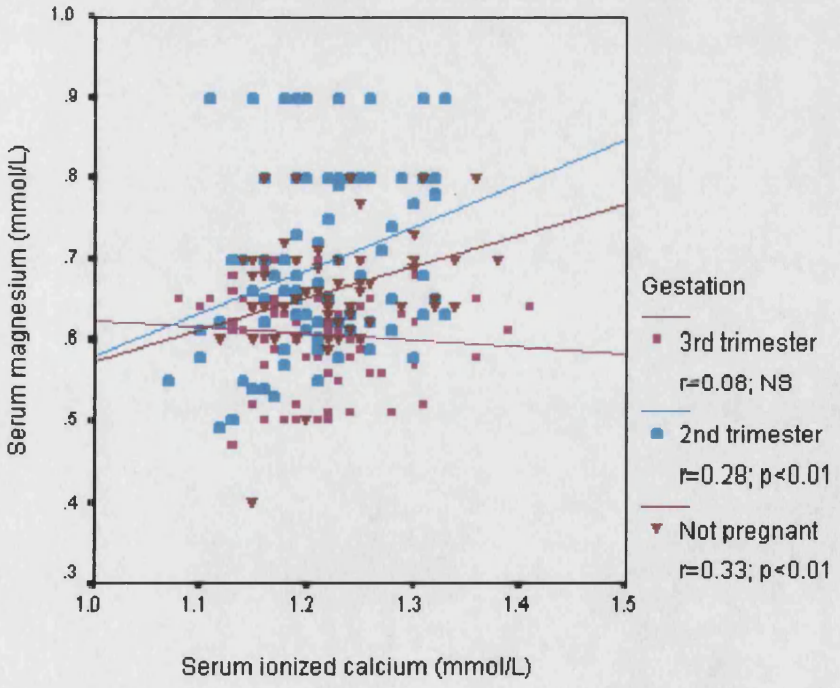
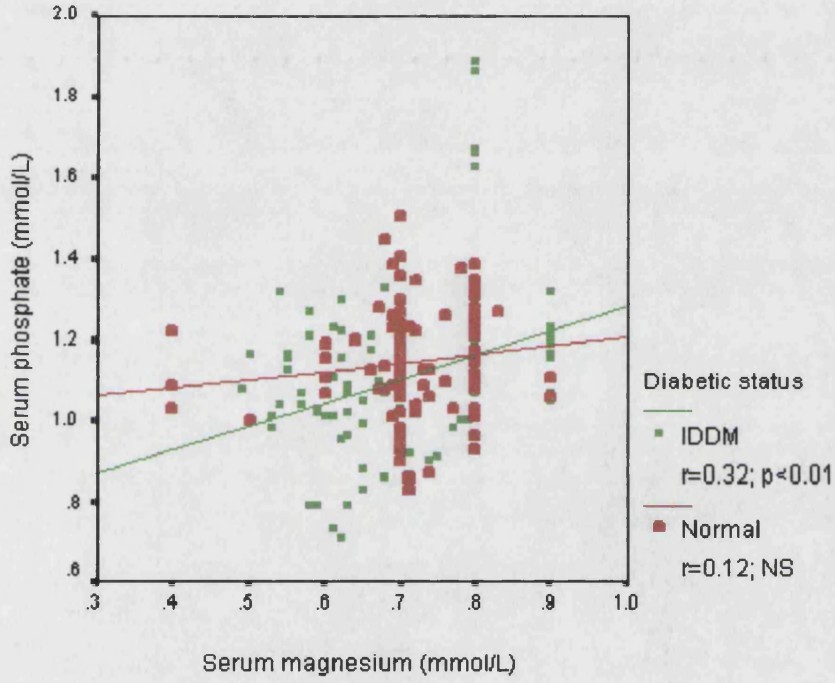


Fig. 3.24 Correlations between serum Mg and serum iCa^{2+} in normal (upper graph) and IDDM (lower graph) women at varying gestations.

In the normal women there was no correlation between serum Mg and serum PO_4 at any gestation. In the IDDM women there was a positive correlation between these two cations at 21 weeks' gestation ($r=0.32$, $p<0.01$) but not on any other occasion (fig 3.25).



Normal[PO_4]=1.00(0.12)+0.20(0.16)[Mg]
IDDM[PO_4]=0.69(0.14)+0.60(0.20)[Mg]

Fig. 3.25 Correlation between serum concentrations of Mg and PO_4 in normal and IDDM women at 21 weeks gestation

3.3C Plasma glucose and calcium homeostasis

Plasma glucose concentrations are shown in table 3.28.

Table 3.28 Plasma glucose concentration (mmol/L). Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	3.8(0.1)	3.7(0.1)	4.2(0.1)
	IDDM	6.6(0.8)	5.8(0.7)	10.7(1.1)
1 hour	Normal	4.2(0.3)	5.0(0.3)	3.8(0.3)
	IDDM	10.6(0.9)	8.6(0.7)	12.7(1.2)
2 hour	Normal	4.0(0.2)	4.5(0.2)	4.2(0.2)
	IDDM	10.2(1.0)	9.6(0.7)	13.0(1.3)
3 hour	Normal	3.9(0.1)	4.0(0.2)	4.4(0.2)
	IDDM	8.2(1.1)	7.5(0.6)	10.5(1.5)
4 hour	Normal	4.0(0.1)	3.9(0.1)	4.7(0.1)
	IDDM	6.6(0.8)	5.0(0.4)	8.2(1.4)

In the normal women the meal did not significantly affect plasma glucose concentration either at 21 weeks' gestation or outside pregnancy (ANOVA). At 31 weeks there was a significant effect ($p<0.001$)) with the 1 hour value being significantly higher than the fasting and 4 hour values.

In the IDDM women the standard breakfast had a significant effect on plasma glucose concentration during pregnancy ($p<0.01$ for both gestations) but was not significant outside pregnancy ($p=0.68$) – ANOVA for all.

Simple factorial ANOVA showed that gestation, time after breakfast and diabetic status all had highly significant effects on plasma glucose concentration ($p<0.001$ for all).

The response of plasma glucose to the standard breakfast at 21 weeks' gestation is shown in fig. 3.26.

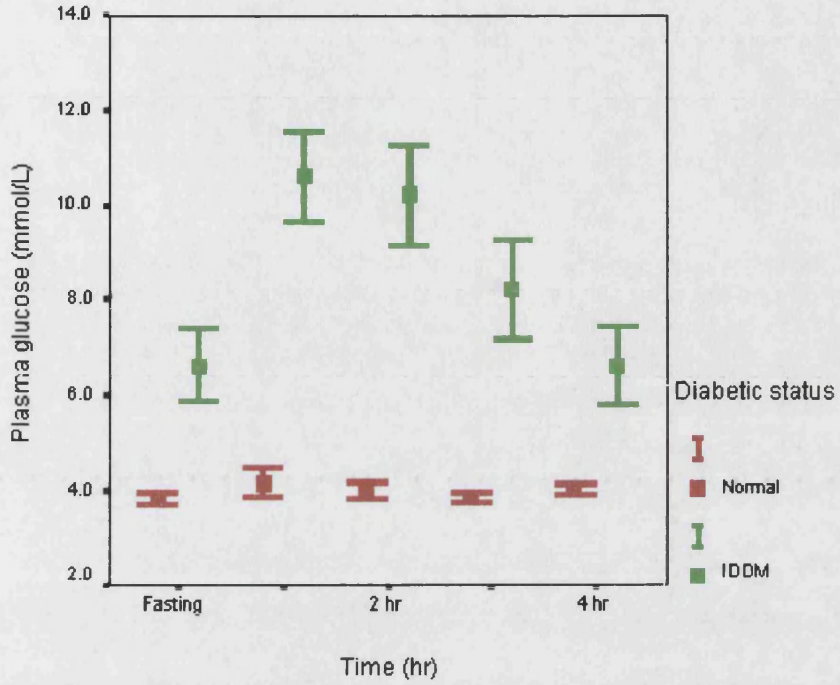
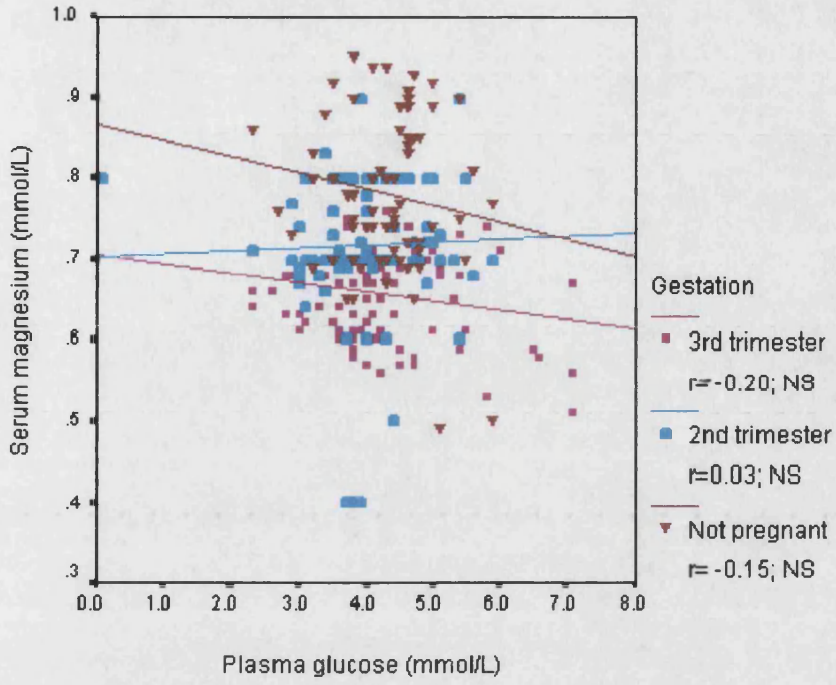


Fig. 3.26 Plasma glucose concentration following the standard breakfast at 21 weeks'gestation in normal and IDDM women. Mean ± SEM.

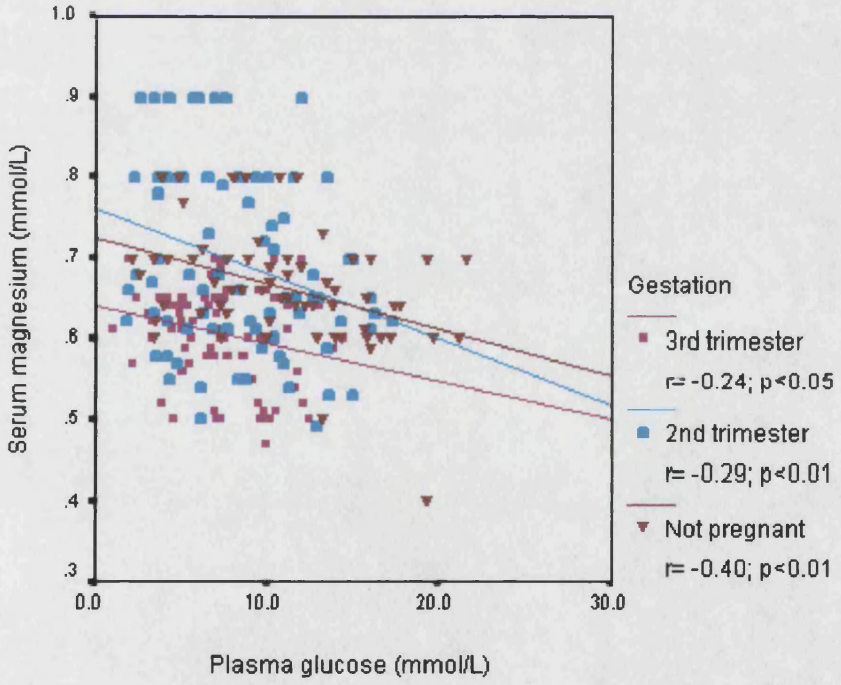
Correlations between plasma glucose and serum iCa^{2+}, Mg and PO_4

Plasma glucose did not correlate with serum iCa^{2+} concentration in either group of women at any gestation.

In the normal women there was no correlation between plasma glucose and either serum Mg or serum phosphate concentrations (figs. 3.27 & 3.28). In the IDDM women at 21 weeks serum glucose was significantly negatively correlated with serum Mg ($r= -0.29$; $p<0.01$) and serum phosphate ($r=-0.60$, $p<0.001$). At 31 weeks' gestation the negative correlation was still apparent for Mg ($r=-0.24, p<0.05$) and PO_4 ($r=-.0.41, p<0.001$). In the non-pregnant IDDM women plasma glucose did not correlate with serum PO_4 ($r=0.09$, NS) but did have a significant negative correlation ($r=-0.40$, $p<0.01$) with serum Mg concentration (figs3.27 & 3.28).

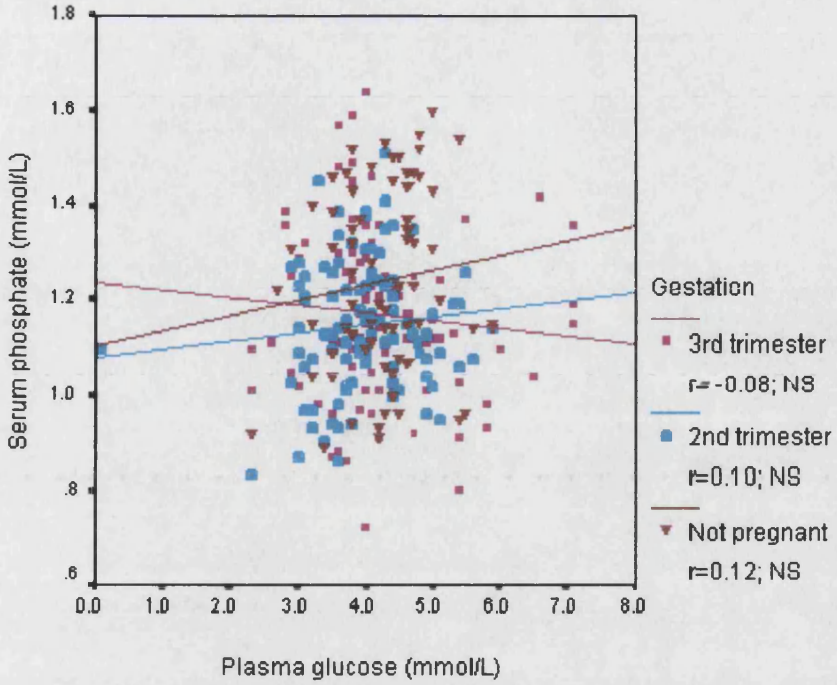


Non-pregnant [Mg] = 0.87(0.07)-0.06(0.02)[glu]
2nd trimester [Mg] = 0.70(0.04)+0.01(0.01)[glu]
3rd trimester [Mg] = 0.70(0.03)-0.03(0.06)[glu]

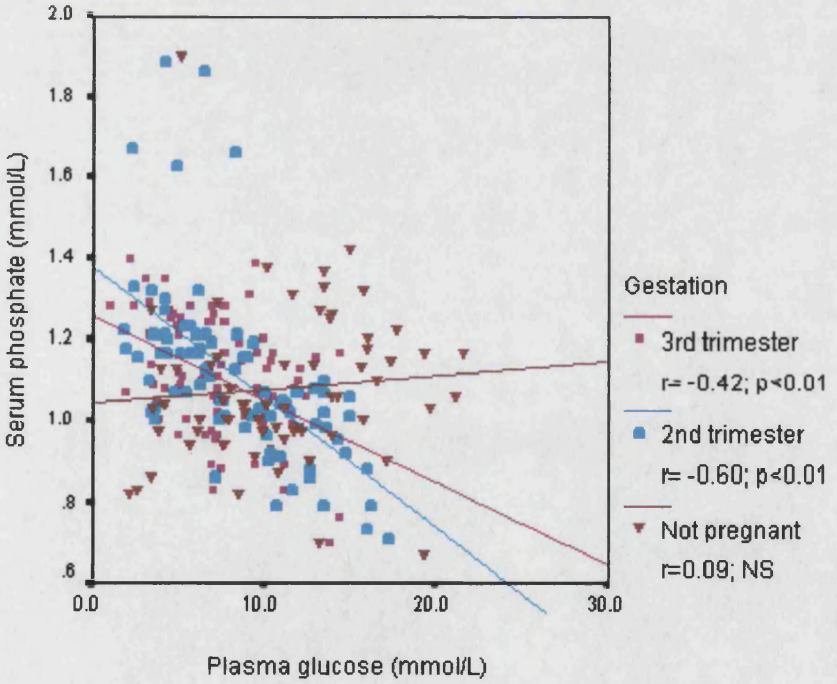


Non-pregnant [Mg] = 0.73(0.02)-0.02(0.01)[glu]
2nd trimester [Mg] = 0.76(0.03)-0.02(0.01)[glu]
3rd trimester [Mg] = 0.64(0.02)-0.02(0.01)[glu]

Fig. 3.27 Correlations between plasma glucose and serum Mg in normal (upper graph) and IDDM (lower graph) women at different gestations



Non-pregnant $[PO_4] = 1.11(0.14)+0.09(0.03)[glu]$
2^{nd} trimester $[PO_4] = 1.08(0.07)+0.05(0.02)[glu]$
3^{rd} trimester $[PO_4] = 1.24(0.09)-0.04(0.02)[glu]$



Non-pregnant $[PO_4] = 1.04(0.06)+0.01(0.01)[glu]$
2^{nd} trimester $[PO_4] = 1.38(0.05)-0.09(0.01)[glu]$
3^{rd} trimester $[PO_4] = 1.25(0.04)-0.05(0.01)[glu]$

Fig. 3.28 Correlations between plasma glucose and serum phosphate in normal (upper graph) and IDDM (lower graph) women at different gestations

3.3D Urinary excretion of creatinine and electrolytes

i Creatinine clearance

Table 3.29 shows creatinine clearance results in both groups of women immediately before and after the standard breakfast and oral Ca load. The fasting values in these tables have been calculated from the hourly aliquots of urine collected in the laboratory on the morning of the calcium load experiment and differ slightly from the creatinine clearances calculated from the 24 hour urine collections made by the volunteers (Table 3.15). However when comparing the effect of the meal it is more appropriate to use these hourly creatinine clearance results as they were collected under identical conditions to the subsequent samples.

Table 3.29 Creatinine clearance (ml/min) in normal and IDDM volunteers. Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	162.1(16.4)	189.4(15.5)	138.6(15.8)
	IDDM	117.8(4.6)	174.1(17.9)	140.6(16.9)
1 hour	Normal	149.2(12.3)	174.1(17.9)	156.2(18.4)
	IDDM	141.6(10.9)	135.7(6.9)	129.2(8.9)
2 hour	Normal	142.0(8.4)	163.1(9.0)	117.4(8.8)
	IDDM	154.4(12.4)	150.6(18.3)	117.0(11.9)
3 hour	Normal	171.8(21.3)	157.5(10.2)	126.7(9.3)
	IDDM	134.1(8.2)	136.9(9.8)	121.1(9.4)
4 hour	Normal	145.0(5.9)	158.3(11.6)	125.1(7.4)
	IDDM	121.5(8.1)	140.4(7.9)	121.0(8.3)

Oral calcium loading and the standard breakfast had no effect on creatinine clearance in either group of women at any of the three time periods (ANOVA).

ii Sodium and potassium excretion

FE sodium rates for both the normal and IDDM women before and after the standard meal are shown in Table 3.30. As mentioned in section 3.2D, calcium supplement status had no effect on FE sodium on the morning of the experiment, therefore the 31 week data are presented and analysed as a unit.

Table 3.30 FE sodium (%) in normal and IDDM volunteers before and following the breakfast and calcium load. Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	0.68(0.07)	0.53(0.05)	0.59(0.07)
	IDDM	0.89(0.10)	0.78(0.07)*	0.57(0.09)
1 hour	Normal	0.73(0.05)	0.51(0.06)*	0.72(0.13)
	IDDM	0.96(0.15)	0.70(0.04)	0.72(0.11)
2 hour	Normal	0.88(0.06)	0.57(0.08)*	0.87(0.09)
	IDDM	1.24(0.11)	0.79(0.08)	1.02(0.15)
3 hour	Normal	1.07(0.06)	0.66(0.07)	1.15(0.09)
	IDDM	1.40(0.12)	0.83(0.06)	1.21(0.13)
4 hour	Normal	1.18(0.09)	0.76(0.10)	1.21(0.12)
	IDDM	1.19(0.13)	0.76(0.06)	1.34(0.14)

*$p<0.05$ comparing normal and IDDM

In general the FE sodium increased after the standard breakfast and calcium load in both groups of women (fig. 3.29 & fig. 3.30). ANOVA was performed to analyse whether time from the breakfast and oral calcium had a significant effect on FE sodium. In the non-pregnant women this was highly significant for both the normal and IDDM women ($p<0.001$) for both. A similar significant effect of time was seen at 21 weeks ($p<0.001$ in the normal women and $p<0.01$ in the IDDM group; ANOVA). However at 31 weeks although visual inspection of the data (fig. 3.30) does show an increase in FE sodium after the meal in the normal group this was not statistically significant ($p=0.09$). FE sodium of the IDDM women at 31 weeks was also not affected by the meal ($p=0.68$).

When the FE sodium response to the standard meal was compared between the normal and IDDM women using multiple regression analysis disease status had no effect outside pregnancy but was highly significant at both 21 weeks ($p<0.01$) and 31 weeks ($p<0.001$).

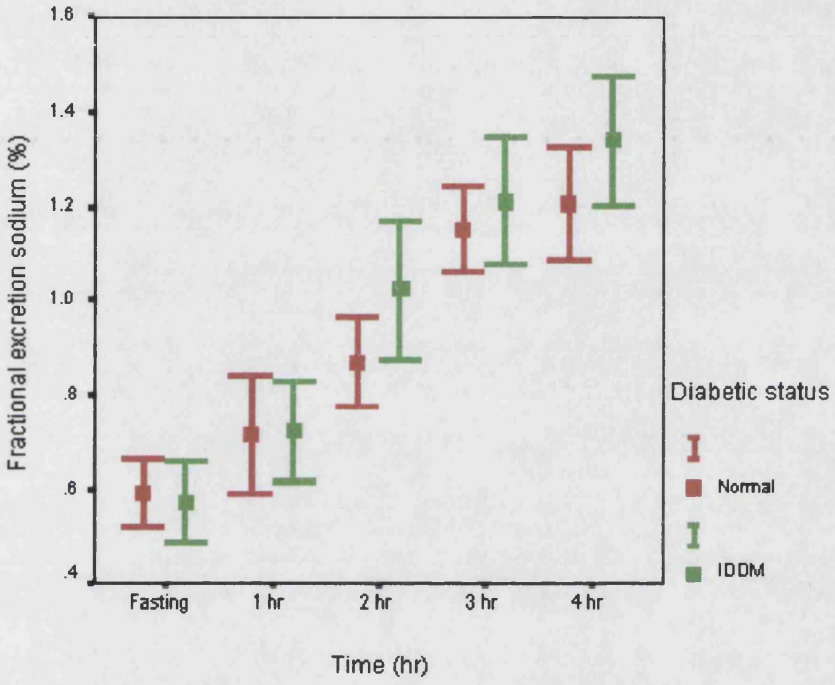
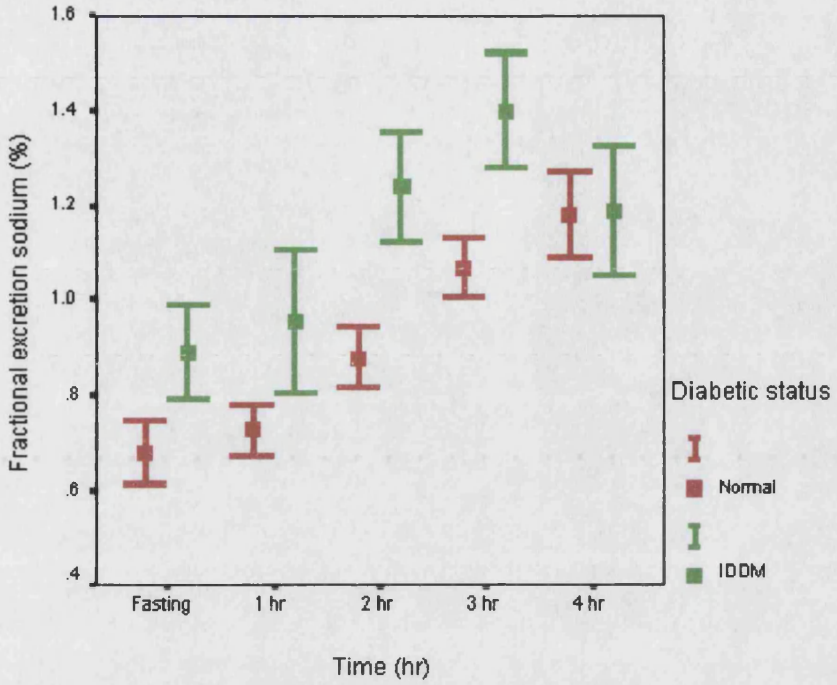


Fig. 3.29 The FE sodium (mean ± SEM) response to the standard meal and oral calcium load in normal and IDDM women outside pregnancy.



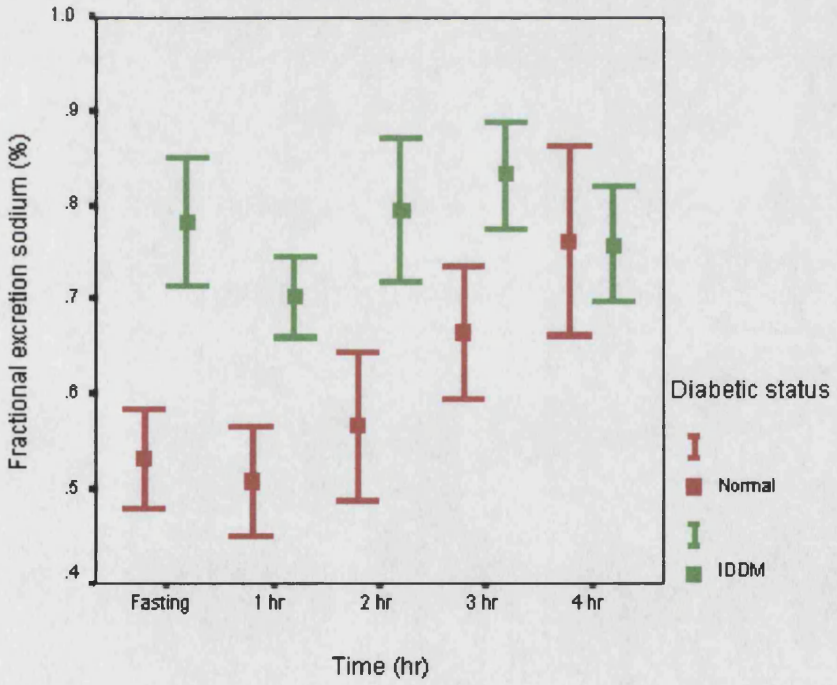


Fig. 3.30 The FE sodium (mean ± SEM) response to the standard meal and oral calcium load in normal and IDDM women at 21 weeks (upper graph) and 31 weeks (lower graph)

When figs 3.29 and 3.30 are compared it can be seen that the FE sodium response to the meal was reduced from the non-pregnant state to 21 weeks and then reduced again by 31 weeks. This pattern was more marked in the IDDM women.

FENa rates for the women who developed PE are shown below in table 3.31. They responded to the meal with a similar pattern of increasing FENa.

Table 3.31 FENa (%) in diabetic women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	0.95	0.65	0.35	0.73	0.61	1.44	0.43	0.74
1 hour	1.06	-	0.33	0.47	0.63	1.36	0.51	1.03
2 hour	1.01	0.92	0.47	1.30	0.66	1.20	0.46	1.06
3 hour	1.46	0.75	0.59	1.37	0.78	1.95	0.85	1.90
4 hour	1.42	0.78	0.55	0.44	0.58	2.27	1.24	1.24

The FE potassium rates at the different time periods are summarized in table 3.32.

Table 3.32 FE potassium (%) in normal and IDDM volunteers before and following the breakfast and calcium load. Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	13.5(1.0)	10.4(1.2)	13.6(1.4)
	IDDM	13.6(1.5)	11.3(1.0)	10.7(1.6)
1 hour	Normal	13.4(1.3)	11.2(1.0)	12.5(1.6)
	IDDM	13.3(1.6)	10.0(0.9)	12.2(1.4)
2 hour	Normal	8.8(0.6)	8.1(1.0)	10.1(1.1)
	IDDM	10.3(1.2)	8.2(1.1)	11.2(1.6)
3 hour	Normal	14.8(1.5)	10.7(0.9)	14.0(1.1)
	IDDM	14.4(1.4)	11.2(1.5)	13.7(1.3)
4 hour	Normal	22.6(1.9)*	17.5(1.2)*	18.1(1.0)
	IDDM	15.9(1.1)	12.9(1.0)	17.0(2.3)

$p<0.01$ comparing normal and IDDM

Outside pregnancy the FE potassium response to the meal and oral calcium load was the same in the normal and IDDM women (fig.3.31). ANOVA was performed and confirmed a significant effect of time from the meal on FE potassium ($p<0.01$ for the normal women and $p<0.05$ for the IDDM women). In the normal women the 2hr data were significantly different to both the fasting and 4hr results whereas for the IDDM women only the 4hr results were different to the other values (post-hoc ANOVA by Scheffe $p< 0.01$).

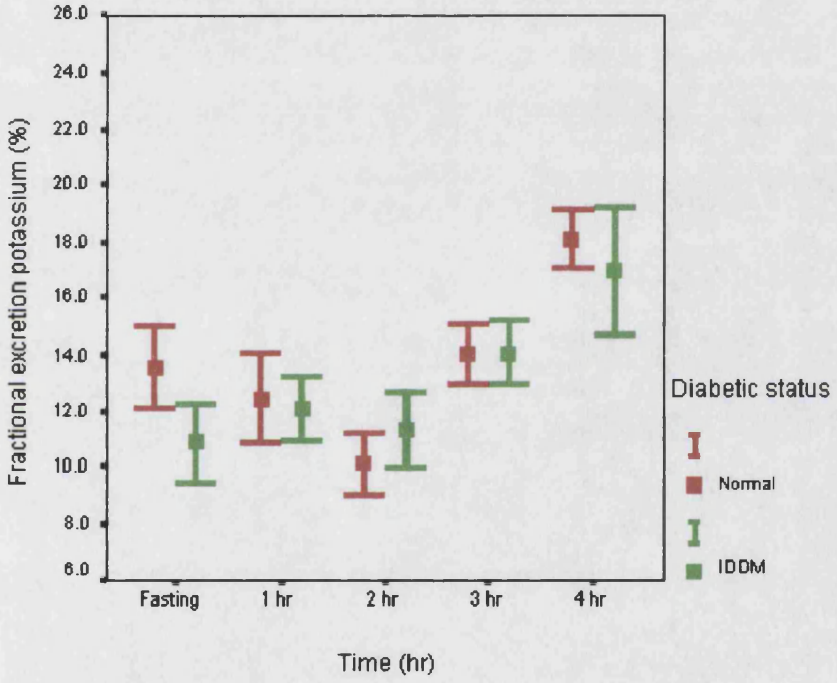
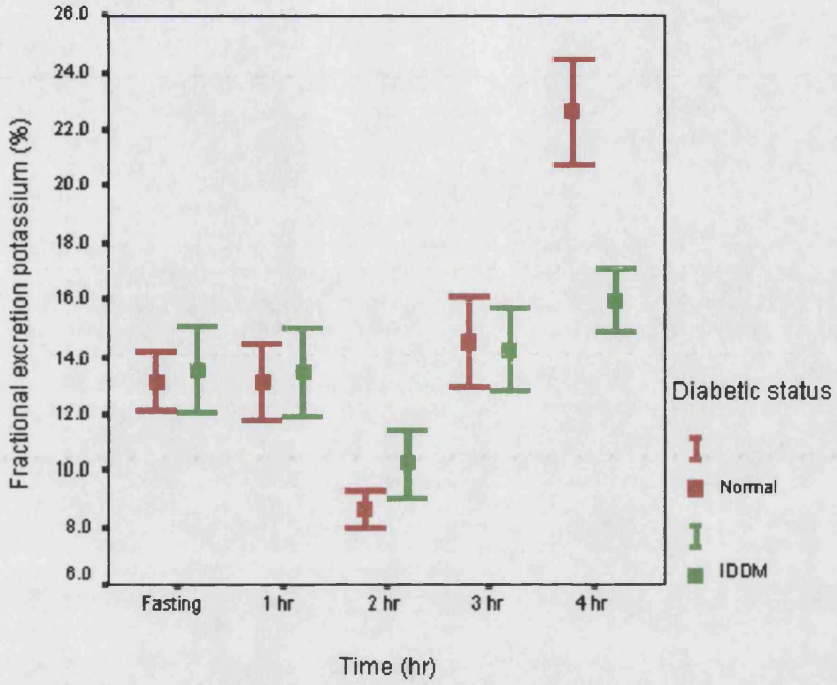


Fig.3.31 The FE potassium (mean/SEM) response to the standard meal and oral calcium in normal and IDDM women outside pregnancy

During normal pregnancy ANOVA did suggest that time after the meal had a significant effect on the FE potassium ($p<0.001$ for both 21 and 31 weeks). Post hoc analysis showed that the only results to be significantly different were the 4hr values. In IDDM pregnancy FE potassium did not show a significant response to the meal and oral calcium (fig. 3.32).



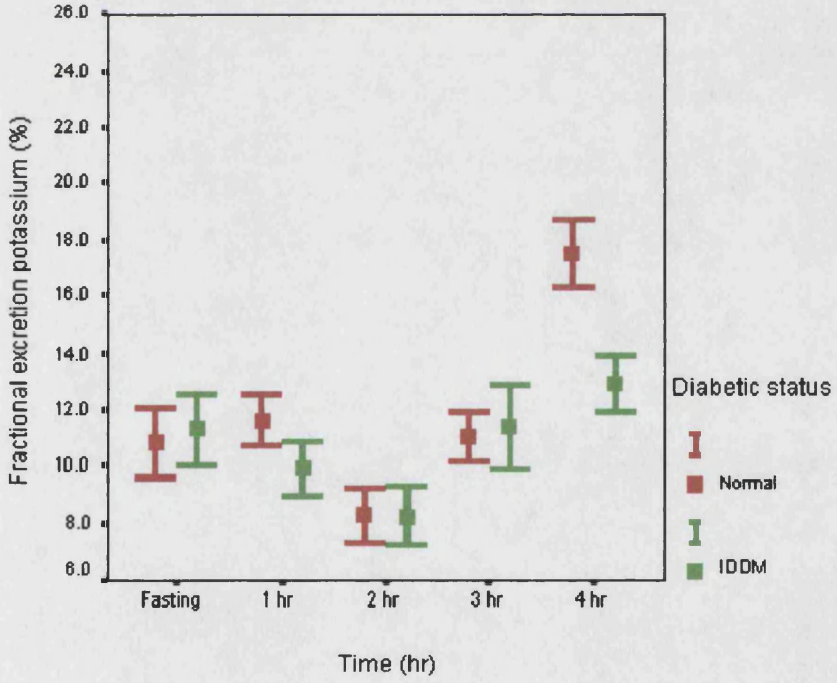


Fig. 3.32 The FE potassium (mean ± SEM) response to the standard meal and oral calcium load in normal and IDDM women at 21 weeks (upper graph) and 31 weeks (lower graph)

The FEK rates in the women who subsequently developed PE are shown in table 3.33. D3 had lower FEK rates at all times but the other two women did not exhibit any consistent pattern when compared with the rest of the IDDM women.

Table 3.33 FEK (%) in diabetic women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	11.95	8.89	4.36	25.76	10.41	26.42	4.12	8.59
1 hour	13.29	-	-	35.21	14.36	23.72	6.82	10.81
2 hour	14.73	10.91	7.53	20.72	12.47	16.52	6.84	8.25
3 hour	16.85	12.62	10.32	27.45	16.70	25.26	8.05	10.94
4 hour	20.39	12.23	12.57	33.58	18.60	22.36	9.76	11.28

Correlations between the FE sodium and FE potassium

Outside pregnancy there was a significant positive correlation between the FE of these two cations in both the normal ($r=0.27$; $p<0.05$) and IDDM women ($r=0.39$; $p<0.01$) – see fig. 3.33. During pregnancy the situation was confused. At 21 weeks there was no correlation in the normal women ($r=0.16$; $p=0.12$) but there was in the IDDM women ($r=0.56$; $p<0.001$); at 31 weeks there was a positive correlation in the normal group ($r=0.31$; $p<0.01$) but not in the IDDM group ($r=0.14$; $p=0.2$).

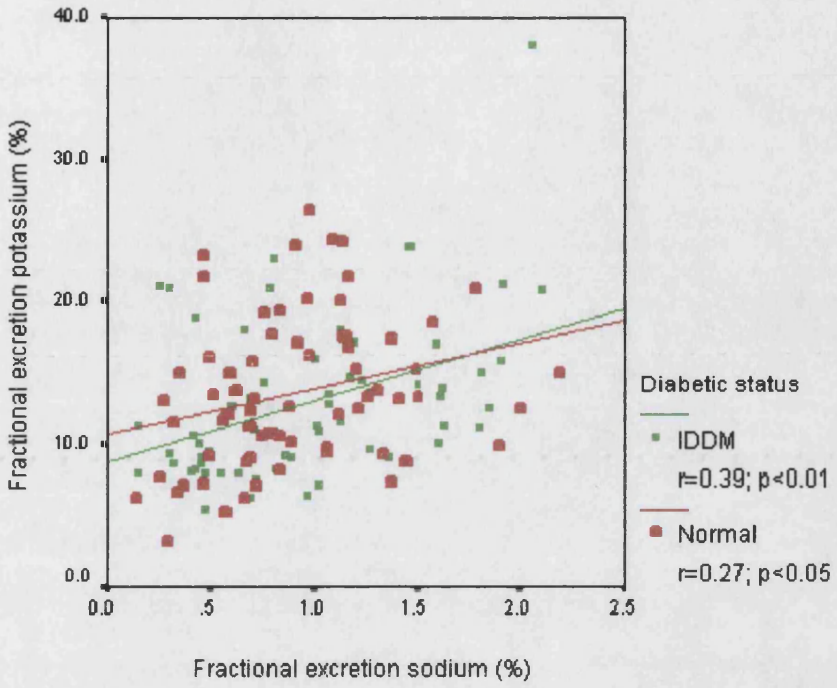


Fig. 3.33 Correlation between FE sodium and potassium in non-pregnant normal and IDDM women

iii Urinary calcium excretion

As mentioned in section 3.2C calcium supplement status had no effect on the excretion of calcium on the morning of the experiment, therefore the 31 week data are presented and analysed as a unit Table 3.34. There was no significant difference between UCE in the normal and IDDM women at any time or gestation.

Table 3.34 Urinary calcium excretion rates (mcmol/min) – median (IQR).

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	2.0(1.0-2.7)	3.2(1.4-6.7)	1.2(0.7-1.5)
	IDDM	3.3(1.6-5.7)	1.7(1.1-4.1)	1.1(0.9-1.7)
1 hour	Normal	3.2(0.9-5.0)	3.2(1.8-6.7)	2.4(1.0-4.9)
	IDDM	3.7(1.7-6.0)	3.2(2.0-4.8)	1.9(1.2-3.5)
2 hour	Normal	7.0(6.4-14.3)	8.6(5.5-12.7)	4.3(2.7-9.4)
	IDDM	8.7(3.8-13.0)	7.0(5.6-8.7)	4.2(3.0-6.2)
3 hour	Normal	8.7(6.4-14.3)	9.7(5.8-17.6)	5.8(3.5-9.5)
	IDDM	8.0(6.3-13.6)	9.5(6.2-12.1)	5.9(3.8-8.1)
4 hour	Normal	8.7(6.3-13.0)	8.6(5.5-15.4)	5.4(4.1-10.4)
	IDDM	10.5(6.0-13.6)	9.8(6.5-12.0)	5.8(5.0-7.7)

In normal volunteers the urinary calcium excretion rate per minute (UCEPM) increased significantly after the oral calcium load at all three times when the women were studied. As UCEPM was not normally distributed values were converted into $\log_{10}$ values and ANOVA could then be performed with post hoc testing by Scheffe at 0.01. At all three gestations ANOVA showed a highly significant ($p<0.001$) effect of time after the calcium load on UCEPM. Post hoc testing showed that significant differences occurred between the fasting and 1hr values, and the hour 2,3 and 4 results. No further rise was apparent in $\log_{10}$UCEPM after hour 3 and values were maintained at this high plateau in the 4^{th} hour (table 3.34 and figs. 3.34 & 3.35).

In the IDDM women UCEPM followed a similar pattern to the normal women (figs.3.34 & 3.35).

Simple factorial ANOVA looking at the combined effects of diabetic status, gestation and time after the oral calcium load on $\log_{10}$UCEPM showed that diabetic status was a significant factor ($p=0.01$) but that time and gestation were more significant ($p<0.001$ for both). Fig. 3.36 shows the effect of gestation on $\log_{10}$UCEPM in the normal and IDDM volunteers.

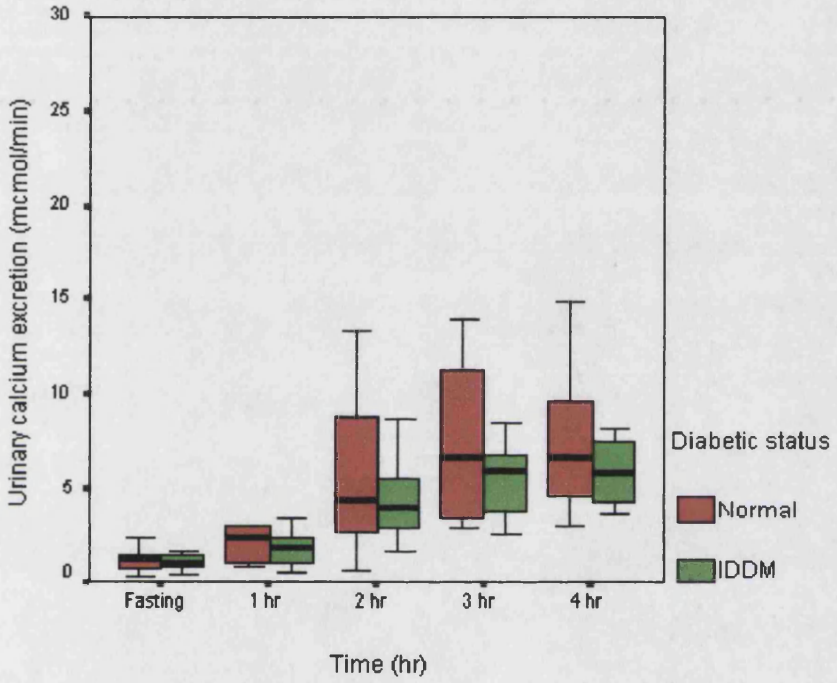
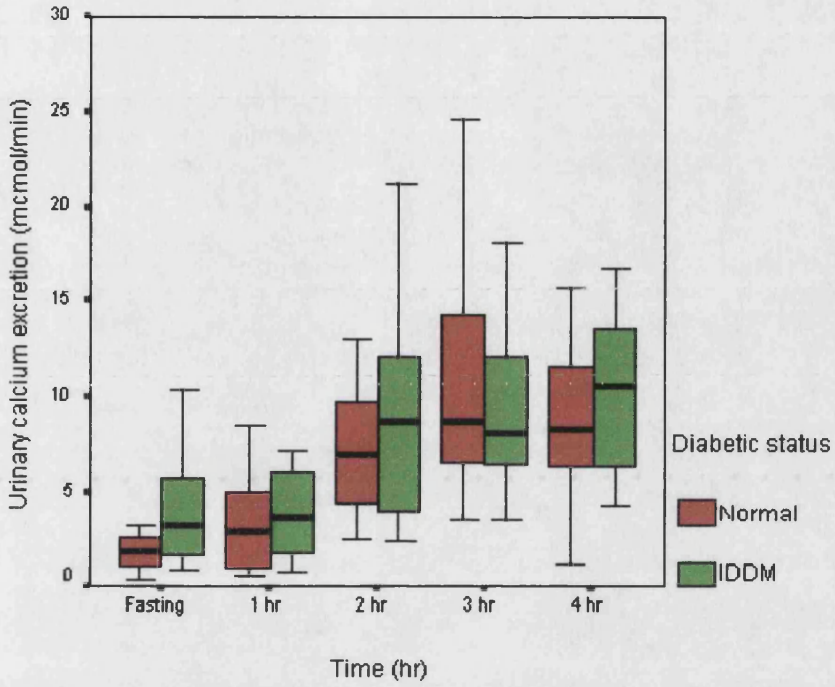


Fig. 3.34 UCEPM before and after the standard breakfast and oral calcium load in the non-pregnant women. Values shown are median (IQR) (absolute range).



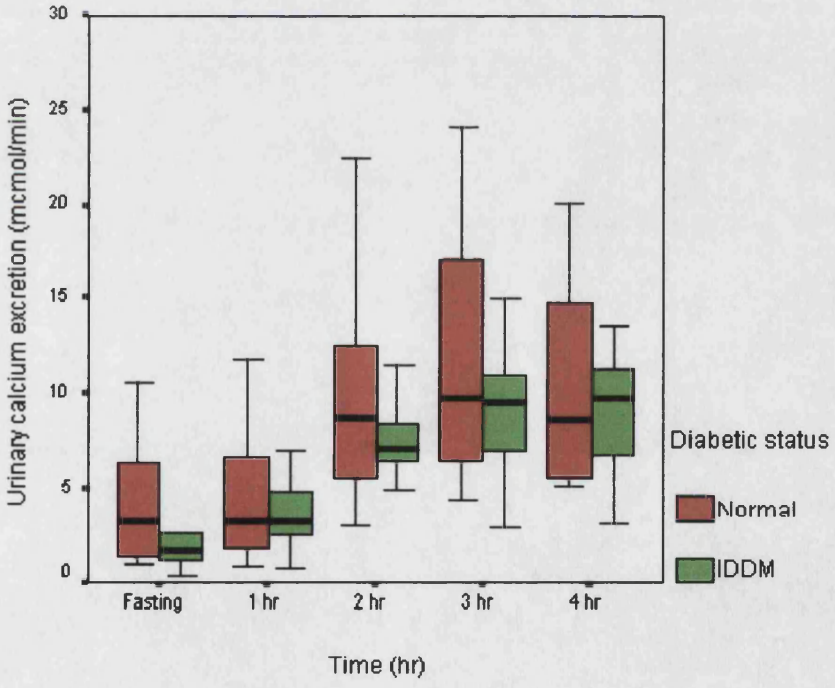
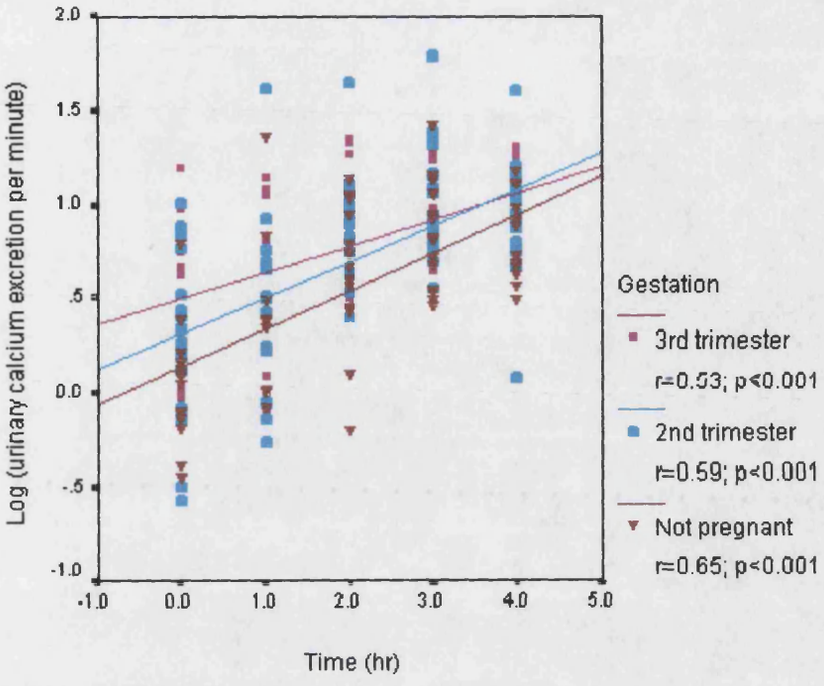
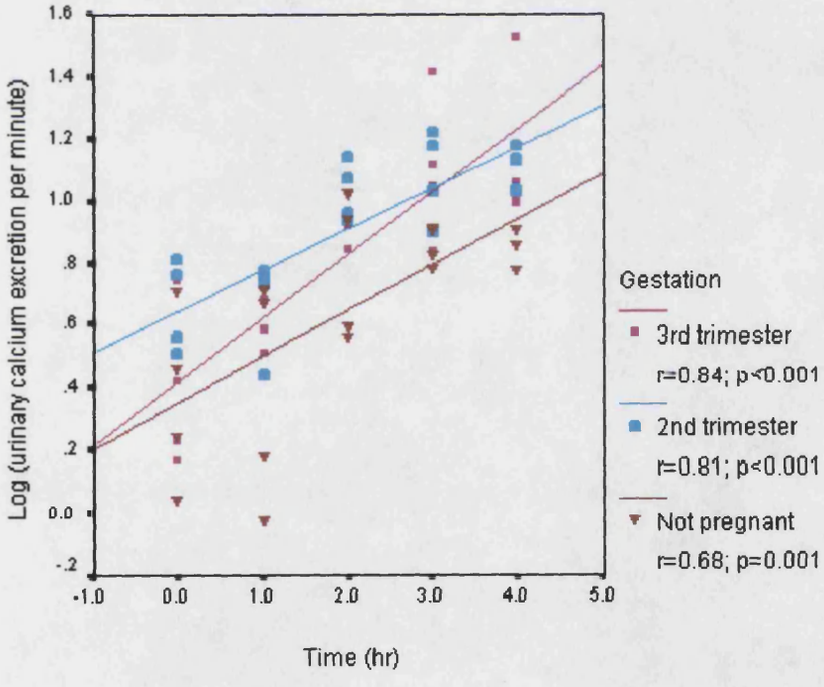


Fig. 3.35 UCEPM before and after the standard breakfast and oral calcium load at 21 weeks (upper graph) and 31 weeks (lower graph). Values shown are median (IQR) (absolute range).



Not pregnant $\log_{10}$(UCEPM) = 0.15(0.07)+0.21(0.03)time
2nd trimester $\log_{10}$(UCEPM) = 0.32(0.07)+0.19(0.03)time
3rd trimester $\log_{10}$(UCEPM) = 0.50(0.06)+0.14(0.03)time



Not pregnant $\log_{10}$(UCEPM) = 0.35(0.09)+0.15(0.04)time
2nd trimester $\log_{10}$(UCEPM) = 0.65(0.06)+0.13(0.02)time
3rd trimester $\log_{10}$(UCEPM) = 0.42(0.08)+0.21(0.03)time

Fig. 3.36 Correlation between UCEPM and time from the oral calcium load in normal (upper graph) and IDDM (lower graph) women at different gestations

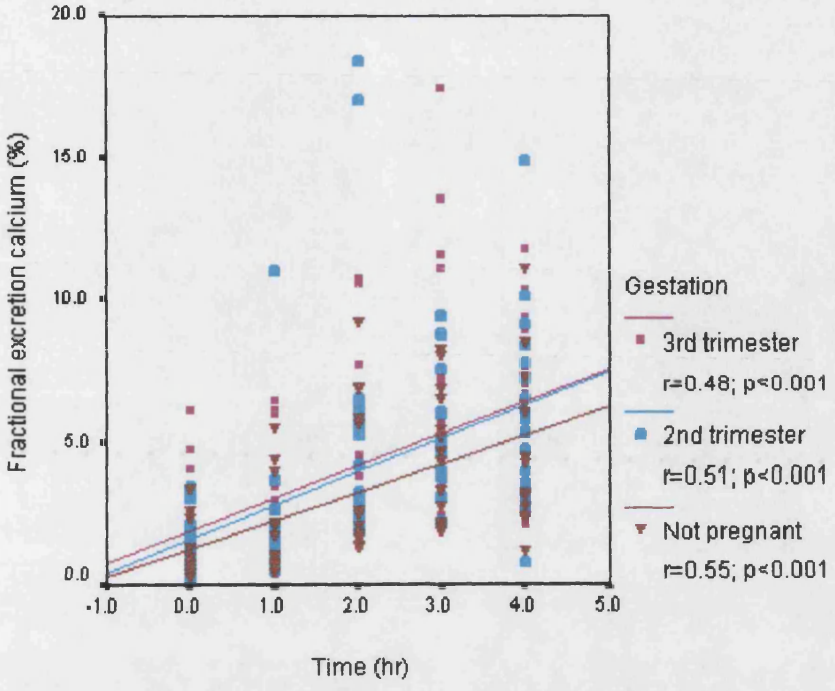
Fractional excretion of calcium rates (FECa) were calculated using the concentration of serum iCa^{2+} rather than the total serum calcium concentration. The FECa on the morning of the experiment was not affected by whether the women were receiving calcium supplements or placebo ($p=0.7$ in normal women and $p= 0.8$ in IDDM women). Therefore the 31 week data were grouped together to simplify analysis (table 3.35)

Table 3.35 FECa (%) in both normal and IDDM women. Mean ± SEM.

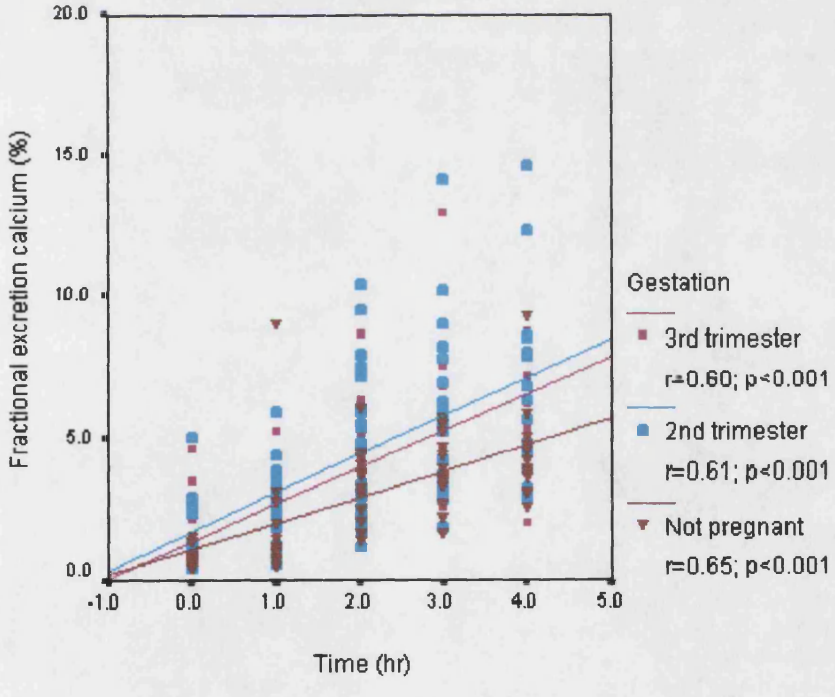
Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	1.34(0.19)	2.02(0.41)	1.09(0.29)
	IDDM	1.77(0.28)	1.54(0.29)	0.97(0.12)
1 hour	Normal	2.23(0.54)	2.33(0.47)	1.92(0.47)
	IDDM	2.49(0.36)	2.07(0.28)	1.54(0.26)
2 hour	Normal	5.47(1.05)	6.21(1.74)	4.04(0.70)
	IDDM	5.09(0.68)	4.32(0.49)	3.53(0.38)
3 hour	Normal	6.57(1.55)	6.61(1.01)	4.56(0.60)
	IDDM	6.18(0.75)	5.79(0.64)	3.97(0.38)
4 hour	Normal	5.88(0.71)	5.58(0.72)	4.75(0.74)
	IDDM	6.71(0.78)	6.19(1.01)	4.14(0.30)

In the normal women at all three gestations ANOVA showed a highly significant effect of time after the calcium load on the FECa ($p<0.001$). The same was true for the IDDM women. Diabetic status had no effect on the FECa response to the calcium load at any gestation (fig 3.37). ANOVA was also performed on both groups of women to see if gestation affected the response to oral calcium but it had no effect (fig. 3.37).

The correlations between the concentrations of serum $iCa^{2+}$and the FECa were significant in both groups of women at all three study times (fig 3.38). There were also significant correlations between serum $iCa^{2+}$and $\log_{10}$ UCEPM (not illustrated). The rate of increase in FECa with increasing serum iCa^{2+} was not affected by disease status but did alter significantly ($p<0.001$ comparing each gestation with the other two) with gestational age. The greatest increase occurred in the 2nd trimester and the smallest in the 3rd trimester. These differences in slopes are shown in fig. 3.38.

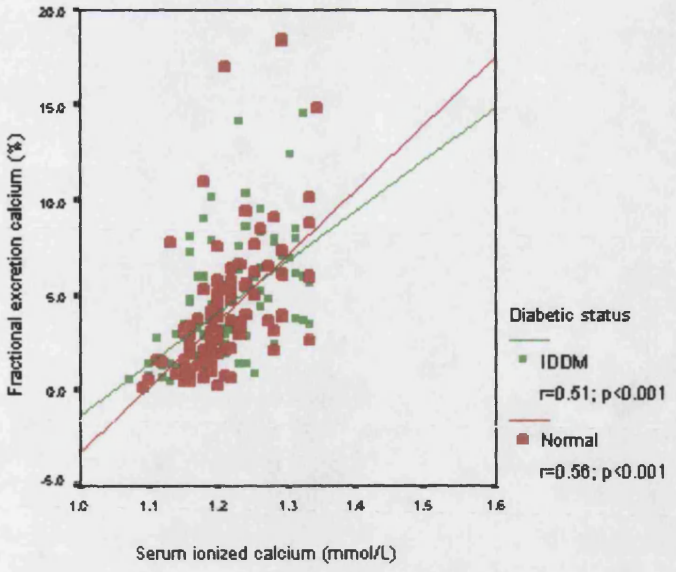


Not pregnant FECa = 1.29(0.46)+0.99(0.19)time
2nd trimester FECa = 1.61(0.51)+1.19(0.21)time
3rd trimester FECa = 1.94(0.55)+1.14(0.22)time

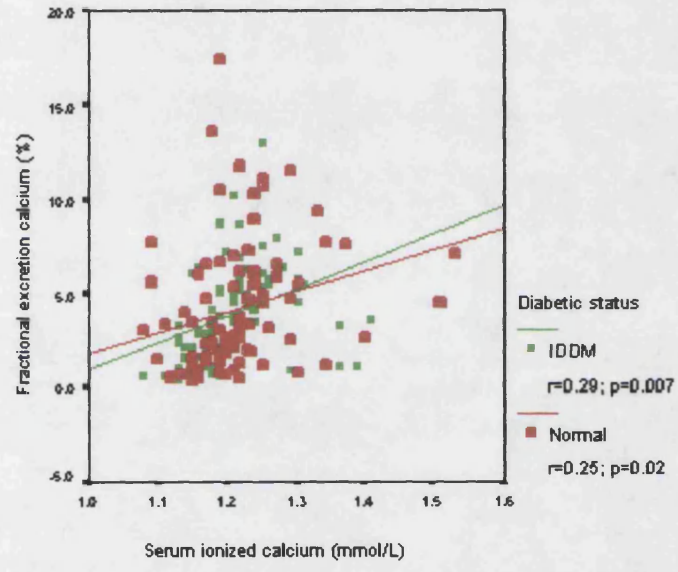


Not pregnant FECa = 1.12(0.33)+0.92(0.14)time
2nd trimester FECa = 1.74(0.47)+1.36(0.19)time
3rd trimester FECa = 1.38(0.46)+1.30(0.19)time

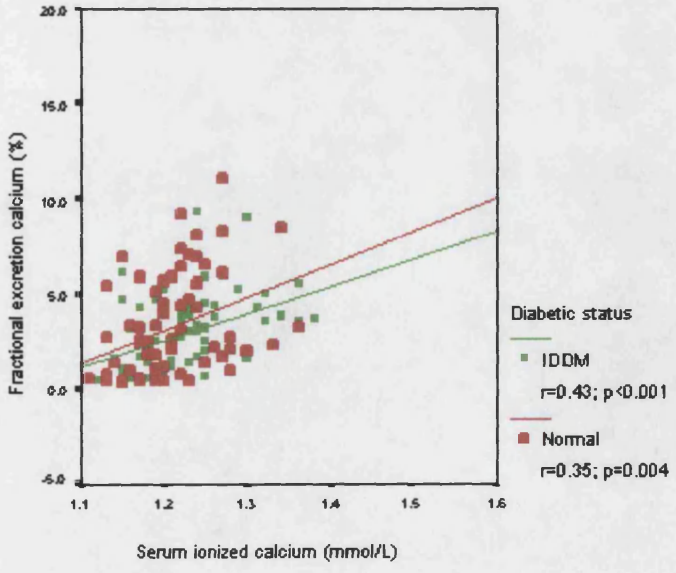
Fig. 3.37 Correlation between FECa and time from the oral calcium load in normal (upper graph) and IDDM (lower graph) women at different gestations



Normal FECa = -38.09(6.51) + 34.83(5.38)iCa^{2+}
IDDM FECa = -28.08(6.04) + 26.83(4.98) iCa^{2+}



Normal FECa = -9.18(5.72) + 11.02(4.69)iCa^{2+}
IDDM FECa = -13.52(6.34) + 14.51(5.24) iCa^{2+}



Normal FECa = -17.65(6.93) + 17.34(5.73)iCa^{2+}
IDDM FECa = -14.46(4.70) + 14.20(3.81) iCa^{2+}

Fig. 3.38 Correlations between FECa and serum iCa^{2+} at 21 weeks (upper graph), 31 weeks (middle graph) and outside pregnancy (lower graph).

Fractional excretion of calcium rates for the women who developed PE are shown in table 3.36. When this is compared with table 3.35 it can be seen that D3 and D5 had raised fasting levels of FECa whilst D10 had levels on the low side. Also D3 largely failed to increase her FECa at any gestation.

Table 3.36 FECa (%) in diabetic women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	5.90	2.60	3.18	4.12	4.34	0.66	0.34	0.22
1 hour	4.02	2.92	2.52	7.61	3.47	0.56	0.12	0.26
2 hour	4.37	4.20	3.91	16.94	11.32	1.04	0.12	0.36
3 hour	7.11	3.30	3.98	19.15	11.80	2.93	0.31	1.68
4 hour	4.61	3.83	3.69	12.15	11.80	4.01	0.59	1.62

iv Urinary phosphate excretion

The fractional excretion of phosphate ($FEPO_4$) rates in both groups of women are shown below in table 3.37. Again this was unaffected by whether the women were receiving calcium supplements or not.

Table 3.37 $FEPO_4$ (%) in both normal and IDDM women. Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	8.8(0.8)*	6.2(0.5)!	8.1(1.7)
	IDDM	12.3(1.0)	11.5(1.3)	11.2(1.6)
1 hour	Normal	10.3(0.8)!	7.9(0.8)!	7.1(2.3)
	IDDM	15.0(1.2)	12.6(1.3)	9.7(1.8)
2 hour	Normal	6.5(0.5)!	6.8(0.9)*	6.2(1.5)
	IDDM	11.1(1.0)	10.6(1.1)	8.7(1.5)
3 hour	Normal	4.4(0.6)!	4.2(0.7)!	5.5(2.1)
	IDDM	8.0(1.0)	8.4(0.9)	5.9(1.4)
4 hour	Normal	7.3(1.8)	3.9(0.6)!	4.8(1.2)
	IDDM	9.0(1.8)	8.1(1.6)	4.4(1.4)

*$p<0.05$ comparing normal and IDDM
!$p<0.01$ comparing norml and IDDM

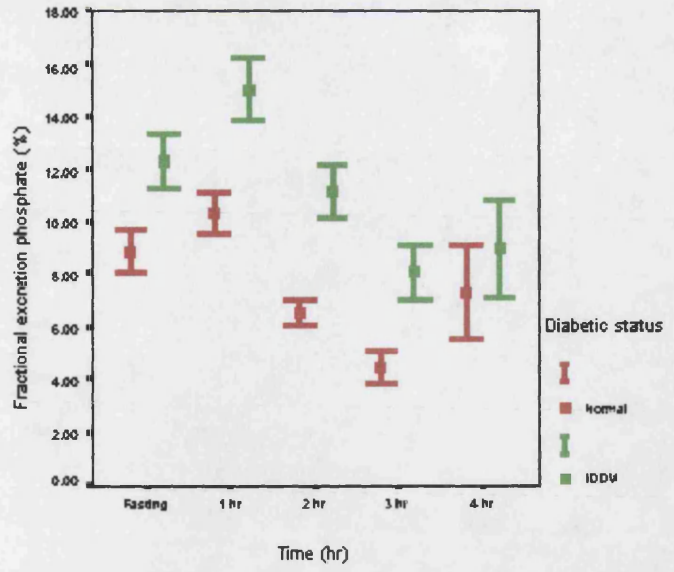
In the normal women at 21 weeks' gestation time after oral calcium loading had a significant effect on $FEPO_4$, (ANOVA, $p<0.001$). Post hoc testing showed that the significant difference was between the peak which occurred at 1hr and at the 3 hr nadir. ANOVA also showed a similar highly significant ($p<0.001$) effect of time on $FEPO_4$ at 31 weeks' gestation. In the non-pregnant normal women the meal and oral calcium load led to a gradual fall in $FEPO_4$ over the next 4hrs, which was not significant (ANOVA, $p=0.8$).

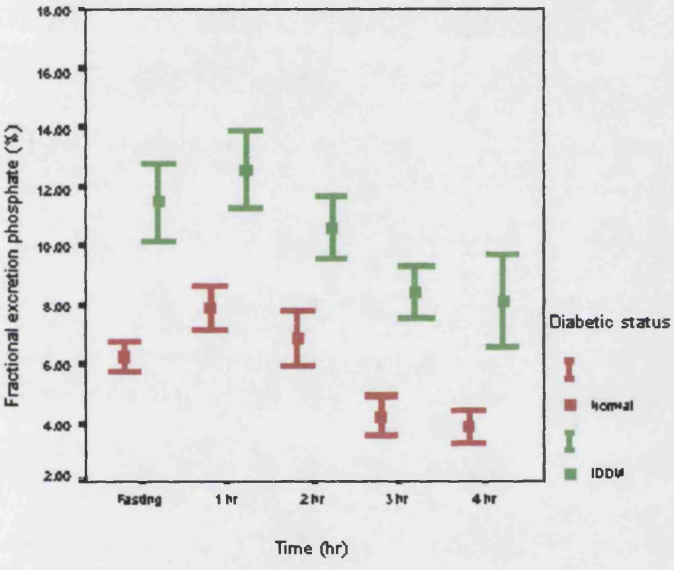
In the IDDM women ANOVA showed a significant effect of time after the meal on $FEPO_4$ at 21 weeks' gestation ($p<0.01$) and outside pregnancy ($p<0.01$). This just failed to reach statistical significance at 31 weeks ($p=0.058$).

It was mentioned above that $FEPO_4$ was significantly higher in the IDDM women than in the normal women during pregnancy. This difference was maintained

throughout the calcium load experiments during pregnancy, except for the final hour of the 21 week experiment when there was no difference between the normal and IDDM women's results (Table 3.37). Outside pregnancy there was no significant difference between the two groups of women. Simple factorial ANOVA of the effect of time after the meal and calcium load on $FEPO_4$ with diabetic status as the co-variate showed that disease status was highly significant ($p<0.001$). Fig. 3.39 highlights the differences between the two groups of women.

In the normal pregnant women there was a significant ($p<0.05$) negative correlation between FECa and $FEPO_4$ rates at 21 weeks but this was not the case for the IDDM women. At 31 weeks again there was a significant ($p<0.05$) negative correlation between the FE rates of these ions in the normal women but the correlation in the IDDM women just failed to reach statistical significance ($p=0.07$). There was no correlation outside pregnancy in either group of women (fig. 3.40).





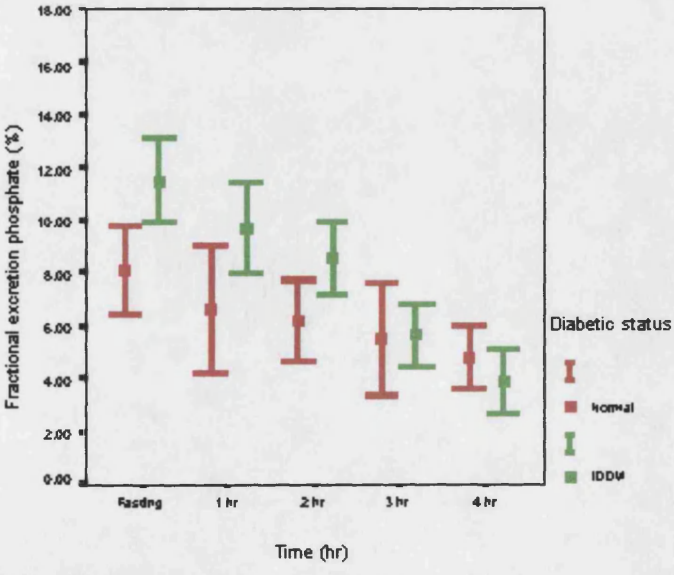
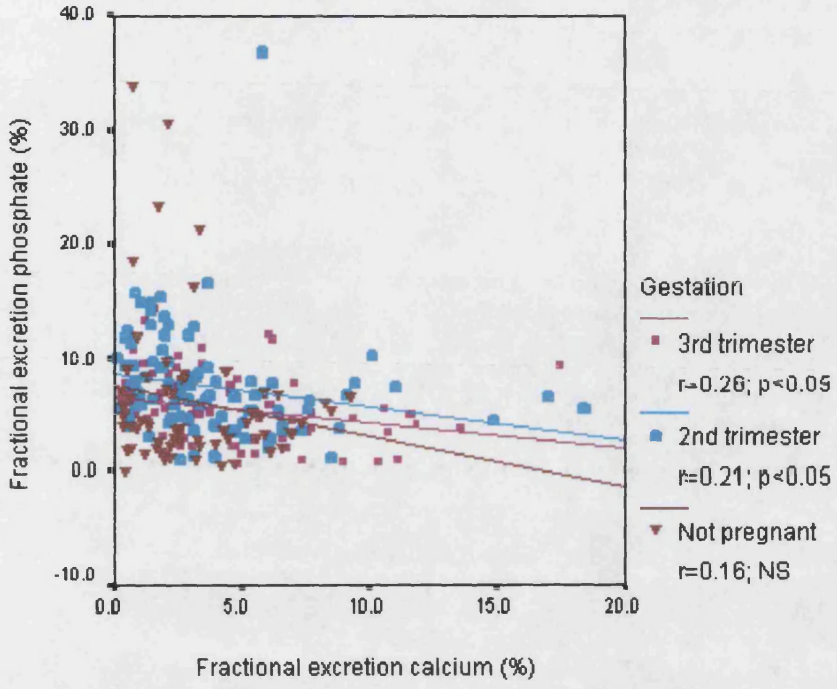
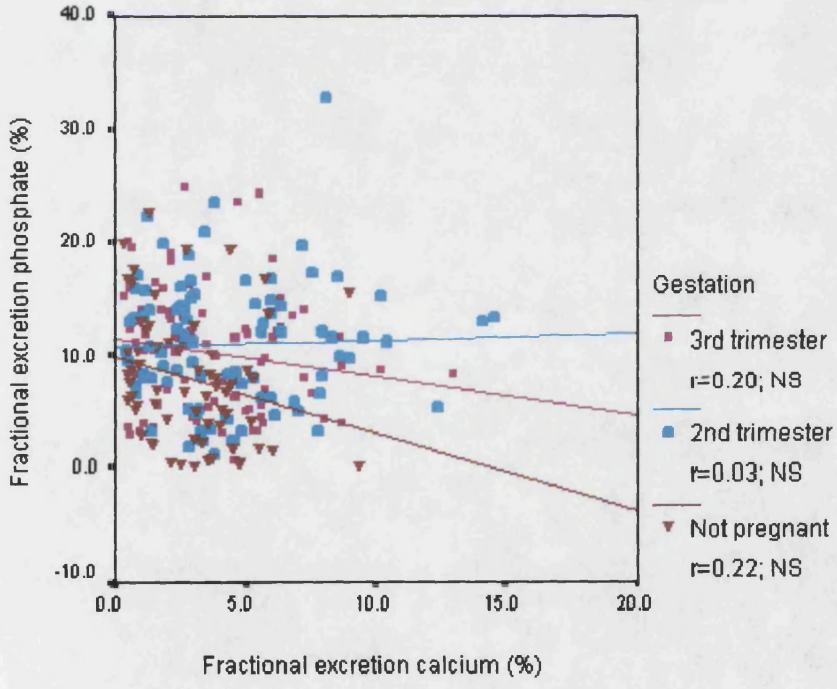


Fig. 3.39 $FEPO_4$ after the oral Ca load at 21 weeks (upper graph), 31 weeks (middle graph) and outside pregnancy (lower graph). Mean ± SEM.



Non-pregnant $FEPO_4 = 7.59(1.33) - 0.45(0.34)FECa$
2^{nd} trimester $FEPO_4 = 8.67(0.75) - 0.29(0.14)FECa$
3^{rd} trimester $FEPO_4 = 6.63(0.49) - 0.23(0.09)FECa$



Non-pregnant $FEPO_4 = 13.08(2.63) - 1.32(0.74)FECa$
2^{nd} trimester $FEPO_4 = 10.83(1.08) + 1.51(0.20)FECa$
3^{rd} trimester $FEPO_4 = 11.59(0.93) - 0.34(0.19)FECa$

Fig. 3.40 Correlations between FECa and $FEPO_4$ in normal (upper graph) and IDDM (lower graph) women at different gestations

$FEPO_4$ rates for the women who developed PE are shown below in table 3.38. It was not possible to see a characteristic pattern in $FEPO_4$ for this group. D3 and D5 tended to have higher $FEPO_4$ rates but even this did not apply at all times. D10 was fairly close to the average results for the other IDDM women at all three times.

Table 3.38 $FEPO_4$ (%) in diabetic women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	14.2	14.6	13.6	22.3	16.5	8.9	9.0	12.9
1 hour	26.1	-	12.4	27.1	15.8	11.2	10.8	9.0
2 hour	18.9	14.4	6.4	49.7	14.9	13.9	13.2	4.85
3 hour	31.5	9.0	2.5	32.0	8.8	12.4	3.5	2.0
4 hour	-	8.8	1.1	2.44	5.37	9.6	11.1	1.4

v Urinary magnesium excretion

Fractional excretion rates of magnesium (FEMg) are shown in table 3.39.

Table 3.39 FEMg (%) in both normal and IDDM women. Mean ± SEM.

Time from meal		21 weeks	31 weeks	Not pregnant
Fasting	Normal	2.18(0.38)	2.26(0.41)	1.93(0.36)
	IDDM	2.56(0.35)	2.32(0.32)	2.93(0.50)
1 hour	Normal	3.12(0.42)	2.43(0.27)	2.44(0.29)
	IDDM	3.42(0.35)	4.75(2.00)	4.03(0.58)
2 hour	Normal	5.66(0.42)	4.88(0.95)	4.52(0.50)
	IDDM	5.63(0.54)	4.64(0.61)	6.55(0.50)
3 hour	Normal	5.91(0.50)	5.10(0.39)	4.80(0.43)
	IDDM	6.98(0.57)	5.95(0.53)	7.37(0.54)
4 hour	Normal	6.70(0.67)	5.41(0.42)	5.17(0.34)
	IDDM	8.49(0.74)	6.97(0.86)	7.75(0.53)

It was mentioned above that there was no difference in basal FEMg between the normal and IDDM women at any gestation. The breakfast and calcium load had a very significant effect (ANOVA, $p<0.001$) on the FEMg which increased in both the normal and IDDM women at all three occasions study gestations. Post hoc analysis showed that FEMg had increased significantly by hour 3 and that hour 4 remained at the same high level. Diabetic status had borderline influence on the response to the meal at 21 weeks gestation (simple factorial ANOVA, $p=0.04$) but not at either 31 weeks or after pregnancy. FEMg response to the meal is shown in fig. 3.41. For clarity only the 21 week data is shown as the response was similar at the other times.

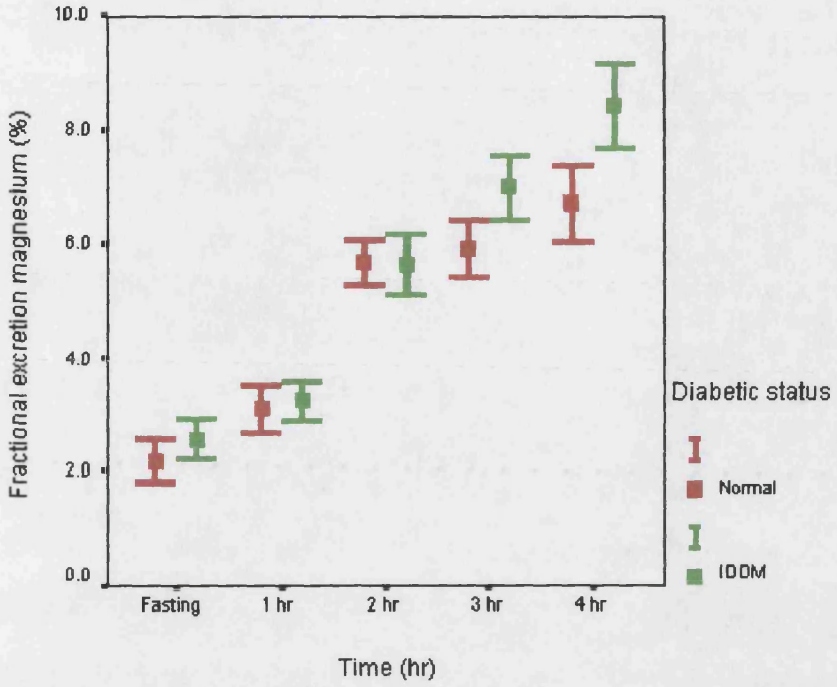


Fig. 3.41 FEMg after the oral Ca load at 21 weeks in normal and IDDM women. Mean ± SEM.

FEMg values for the women who developed PE are shown below (table 3.40). D5 and D10 tended to behave in a similar way to the other IDDM women while D3 had a totally different pattern with virtually no increase in FEMg following the calcium load. This is very similar to her lack of increase in FECa (table 3.36). Also D3 and D5 had high basal levels of FEMg compared to the other IDDM women, again this is similar to their high basal levels of FECa.

Table 3.40 FEMg (%) in diabetic women who developed PE

	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	5.43	4.28	4.48	5.09	3.68	0.63	2.87	3.87
1 hour	4.72	-	2.75	5.87	7.35	1.28	2.25	3.74
2 hour	3.75	4.53	3.07	11.40	8.09	0.99	2.71	4.03
3 hour	5.09	4.03	2.40	15.20	5.52	7.58	6.31	7.13
4 hour	-	4.18	2.96	10.81	8.61	9.73	8.57	7.52

3.4 HORMONE RESULTS

3.4A The effect of calcium loading on serum iPTH

1. Fasting values

In the normal women pregnancy had no significant effect on fasting levels of serum iPTH and neither did calcium supplementation (fig 3.42). It can be seen that the scatter in the non-pregnant group was very large.

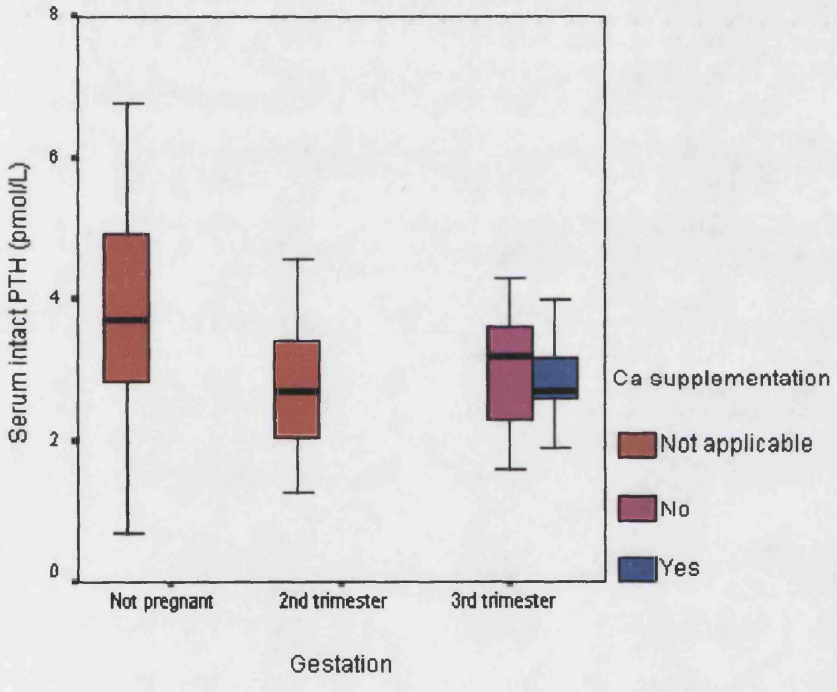


Fig. 3.42 Fasting serum iPTH in the normal women. Values shown are median (IQR) (absolute range).

In the IDDM women pregnancy had a significant effect on iPTH with values being lower at 21 weeks when compared to not pregnant ($p<0.05$, Wilcoxon signed ranks). Calcium supplementation had no effect on fasting iPTH in the IDDM women at 31 weeks. Between 21 and 31 weeks there was a slight rise in fasting iPTH in the diabetic women but this was not statistically significant (fig. 3.43).

Compared to normal controls IDDM women tended to have lower iPTH at all gestations, significant ($p<0.05$) at 21 weeks (fig. 3.44).

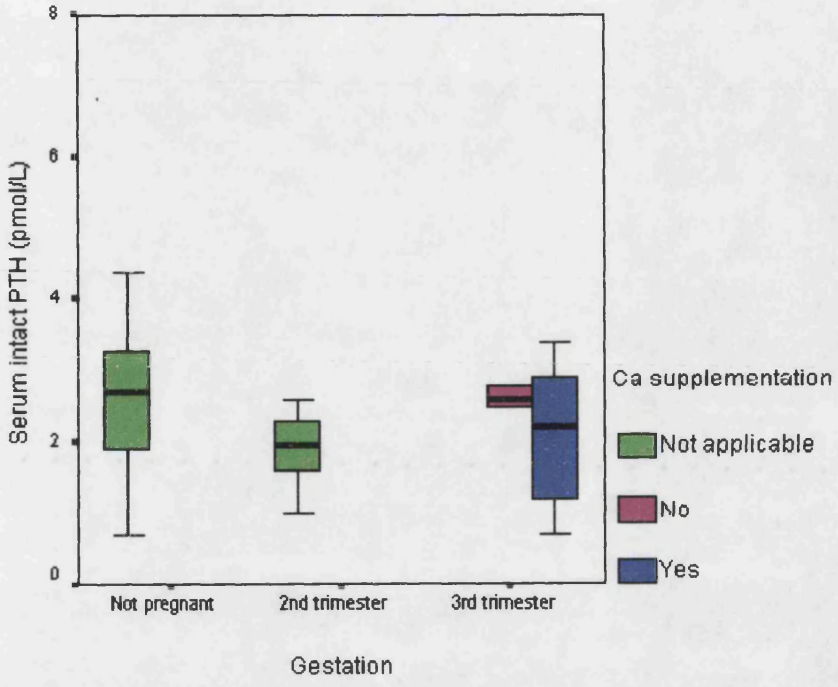


Fig. 3.43 Fasting iPTH in the IDDM women. Values shown are median (IQR) ((absolute range).

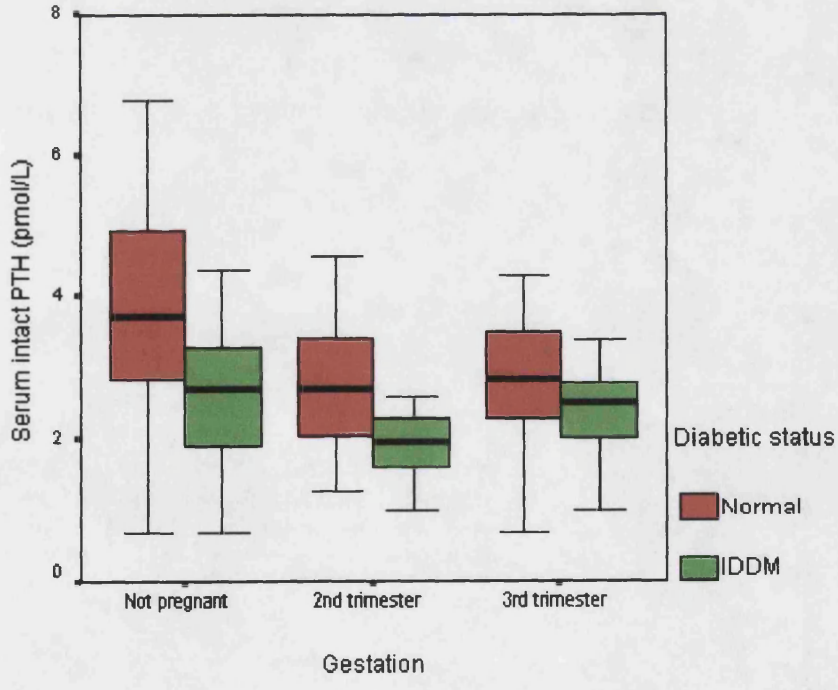


Fig. 3.44 Fasting iPTH in normal and IDDM women. Values shown are median (IQR) (absolute range).

2. Following oral calcium loading

The oral calcium load produced an immediate suppression of iPTH in both groups of women at all three time periods and regardless of whether or not they were on calcium supplements. This suppression was maintained throughout all 4 hours of the experiment. ANOVA was performed on normally distributed $\log_{10}$[iPTH] confirming a very significant effect of the oral calcium loading ($p<0.01$). Post-hoc testing confirmed what visual inspection of the data suggested, namely that the difference occurred between the fasting and 1 hour values in all groups and at all times except for the non pregnant diabetic women in which suppression occurred more slowly, only becoming apparent at 2 hours.

Values of iPTH at all times and gestations are shown in table 3.41. The response of serum iPTH to oral calcium loading is shown graphically in fig. 3.45 – only the 21 week data is shown for clarity as the other time periods were similar.

Table 3.41 Serum iPTH(pmol/l) in normal and IDDM women. Median (IQR)

Time from meal		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
Fasting	Normal	2.7(2.0-3.5)*	2.7(2.3-3.4)	3.5(2.3-4.1)*	3.7(2.7-5.1)
	IDDM	2.0(1.6-2.3)	2.6(2.3-3.5)	2.2(1.1-3.0)	2.7(1.9-3.8)
1 hour	Normal	1.2(0.7-2.0)	1.6(1.4-2.4)	1.2(0-2.1)	1.2(0-2.1)
	IDDM	1.3(0-2.1)	1.6(1.0-2.1)	0(0-1.5)	1.5(0-1.8)
2 hour	Normal	1.4(0.8-1.9)*	1.7(1.4-1.9)	1.3(0-2.3)	2.0(1.1-2.4)
	IDDM	0(0-1.1)	1.6(0-2.4)	0.8(0-1.3)	0.8(0-1.9)
3 hour	Normal	1.5(1.0-1.9)!	1.6(1.1-2.0)	1.4(0.8-1.9)*	2.0(1.3-2.4)!
	IDDM	0.8(0-1.1)	1.3(0.9-2.5)	0.8(0-1.1)	0.9(0-1.3)
4 hour	Normal	1.3(0.7-2.1)*	1.4(0-2.0)	1.3(0-1.5)	1.6(1.1-2.5)
	IDDM	1.0(0-1.3)	1.4(1.1-1.8)	1.0(0-1.3)	0(0-1.7)

*$p<0.05$ comparing normal and IDDM
!$p<0.01$ comparing normal and IDDM

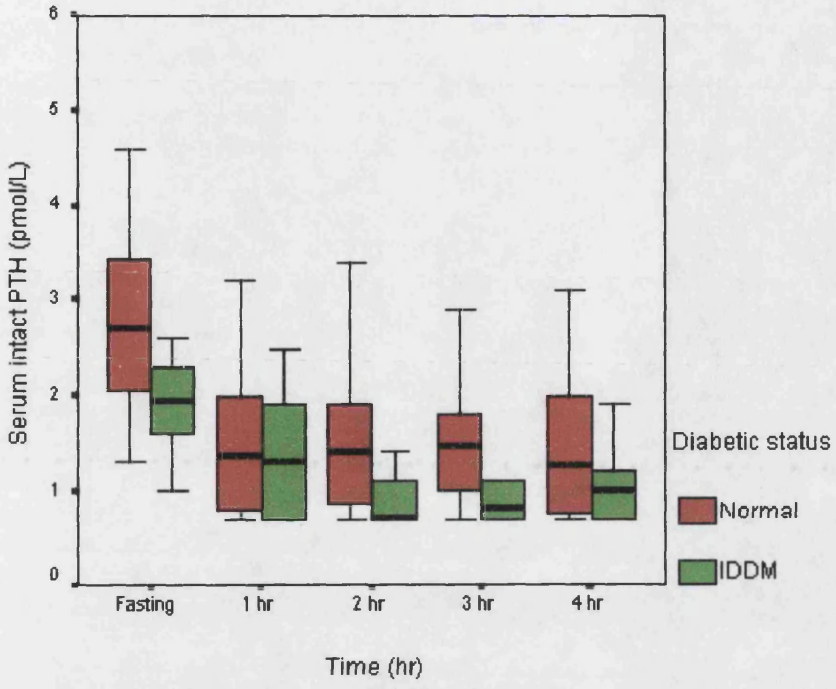
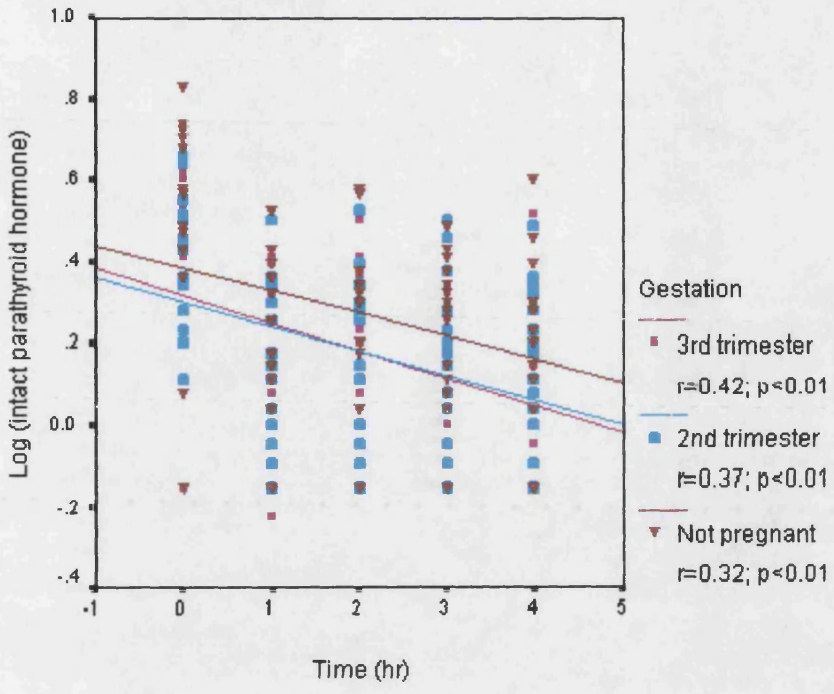
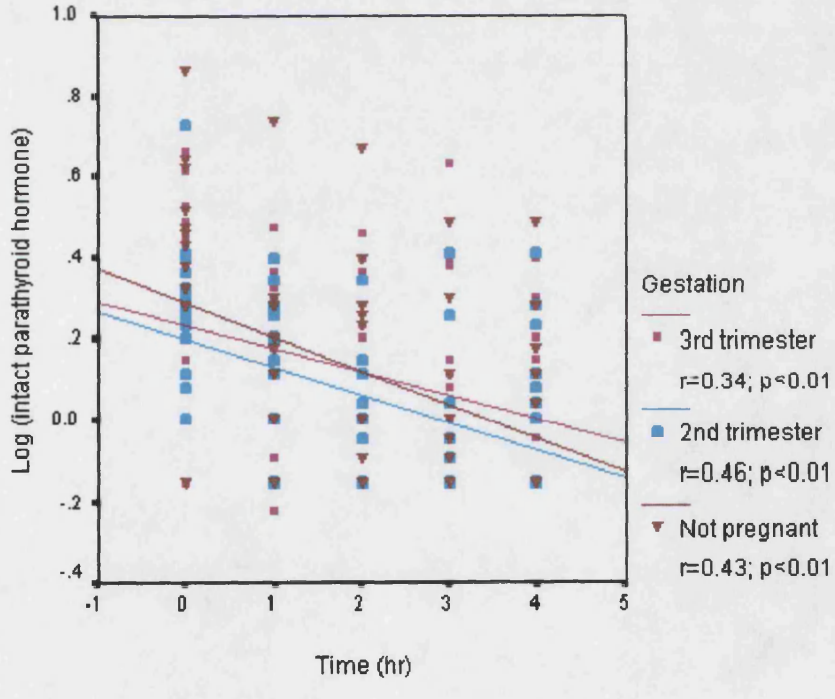


Fig. 3.45 Effect of a standard breakfast and oral calcium loading on serum iPTH at 21 weeks gestation. Values shown are median (IQR) (absolute range).

The correlation of $\log_{10}$[iPTH] with time for both groups of women is shown in fig. 3.46. When the slopes of these graphs were compared by a modified t-test there were all statistically similar showing that neither disease status nor gestation affected the response of serum iPTH concentration to a standard breakfast and oral calcium load.



Not pregnant $\log_{10}$[iPTH] = 0.39(0.05) – 0.15(0.02)time
2nd trimester $\log_{10}$[iPTH] = 0.30(0.04) – 0.17(0.02)time
3rd trimester $\log_{10}$[iPTH] = 0.32(0.04) – 0.18(0.02)time



Not pregnant $\log_{10}$[iPTH] = 0.29(0.06) – 0.23(0.02)time
2nd trimester $\log_{10}$[iPTH] = 0.20(0.04) – 0.19(0.02)time
3rd trimester $\log_{10}$[iPTH] = 0.23(0.04) – 0.12(0.02)time

Fig. 3.46 Correlations between log10[iPTH] and the time from the oral Ca load in normal (upper graph) and IDDM (lower graph) women at different gestations

Results of serum concentrations of iPTH for the IDDM women who developed PE are shown below in table 3.42. In general they followed a similar pattern to above in that there was an immediate suppression of iPTH following the standard breakfast and oral calcium loading which had not returned to baseline at the conclusion of the experiments. It can be seen that D10 had exceptionally high values of iPTH in the 3rd trimester.

Table 3.42 Serum iPTH (pmol/L) in diabetic women who developed PE

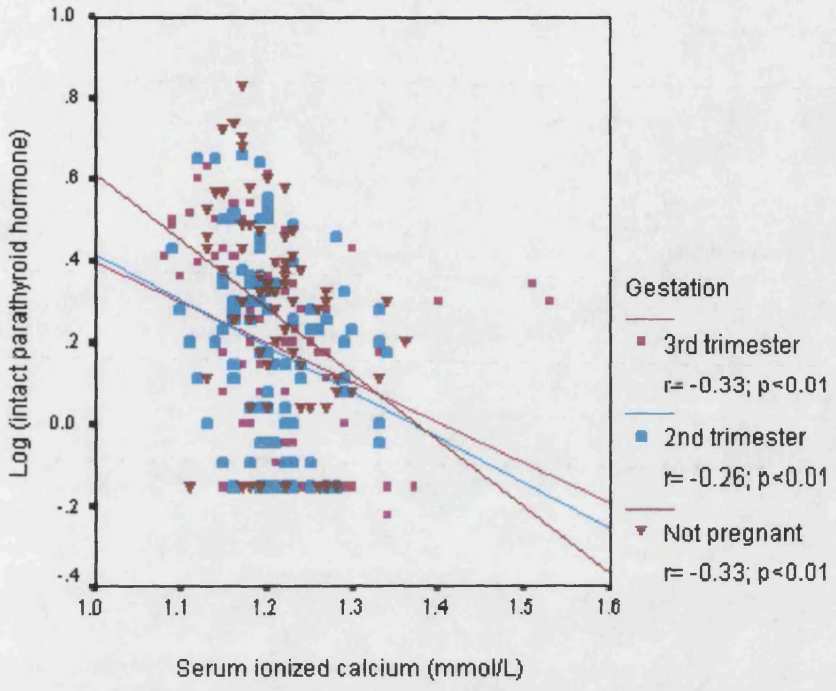
	D3			D5		D10		
	21wk	31wk	NP	21 wk	31wk	21wk	31wk	NP
Fasting	2.3	3.2	3.2	1.1	0.9	2.0	7.6	2.7
1 hour	1.9	1.6	1.7	<0.8	1.2	2.0	N/A	1.7
2 hour	1.7	1.8	1.0	<0.8	<0.8	1.2	3.5	1.8
3 hour	1.1	1.9	1.1	<0.8	1.0	<0.8	3.0	<0.8
4 hour	1.5	2.2	1.3	<0.8	<0.8	1.0	2.4	<0.8

3.4B Correlations between serum iPTH and other variables

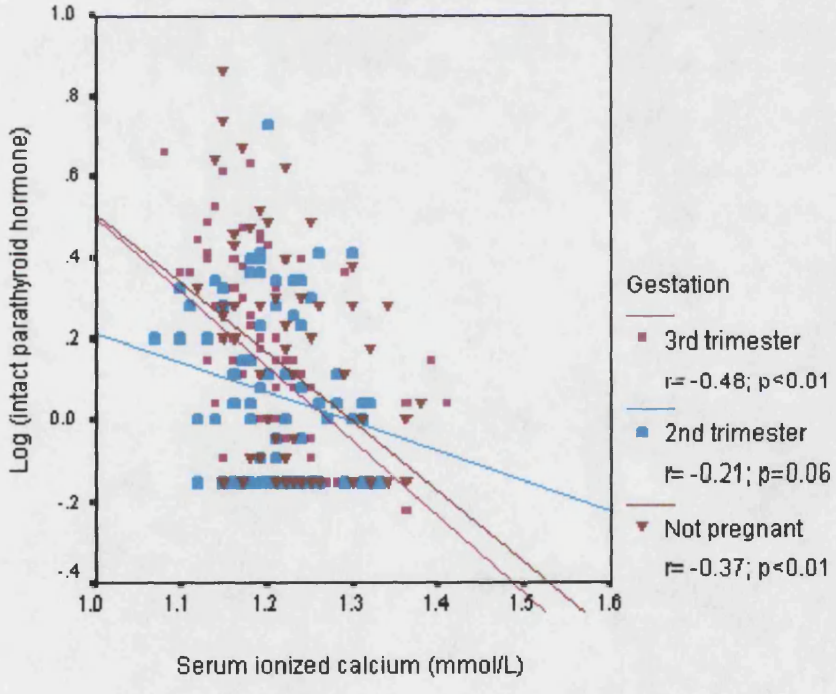
Log_{10}[iPTH] showed significant ($p<0.01$) negative correlations at 21weeks, 31 weeks and outside pregnancy with serum iCa^{2+} in the normal women and at 31 weeks and after pregnancy in the IDDM women ($p<0.01$). At 21 weeks in the IDDM women the correlation just failed to be significant ($p=0.06$) (fig. 3.47).

Outside pregnancy there were significant negative correlations between log_{10}[iPTH] and the concentration of serum PO_4 ($p=0.002$) in both normal and IDDM groups (fig. 3.48) but no correlation during pregnancy.

In the normal women there was no correlation between log_{10}[iPTH] and serum magnesium concentration on any of the three occasions when the women were studied. However the IDDM women exhibited a significant correlation between these two factors at 21 weeks' gestation ($r = -0.35$; $p<0.01$) and outside of pregnancy ($r= -0.29$; $p<0.05$). There was no correlation at 31 weeks' gestation ($r=0.06$; $p=0.6$) (fig. 3.49). As significant correlations also occurred between serum iCa^{2+} and serum Mg in the IDDM women at 21 weeks and outside pregnancy, but not at 31 weeks nor in the normal women (fig. 3.24), partial correlation analysis was used to control for a possible confounding effect of serum iCa^{2+}. Using this there was still a significant negative correlation ($p<0.01$) between serum iPTH and serum Mg in the IDDM women at 21 weeks but not outside pregnancy.

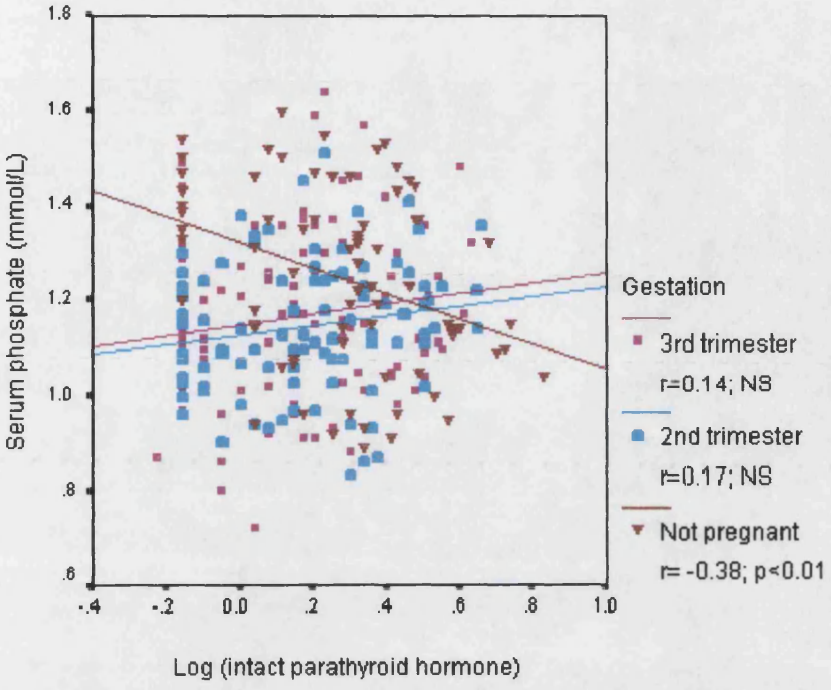


Not pregnant $\log_{10}[iPTH] = 2.26(0.66) - 1.65(0.55)iCa^{2+}$
2^{nd} trimester $\log_{10}[iPTH] = 1.55(0.51) - 1.13(0.42)iCa^{2+}$
3^{rd} trimester $\log_{10}[iPTH] = 1.38(0.37) - 0.98(0.31)iCa^{2+}$

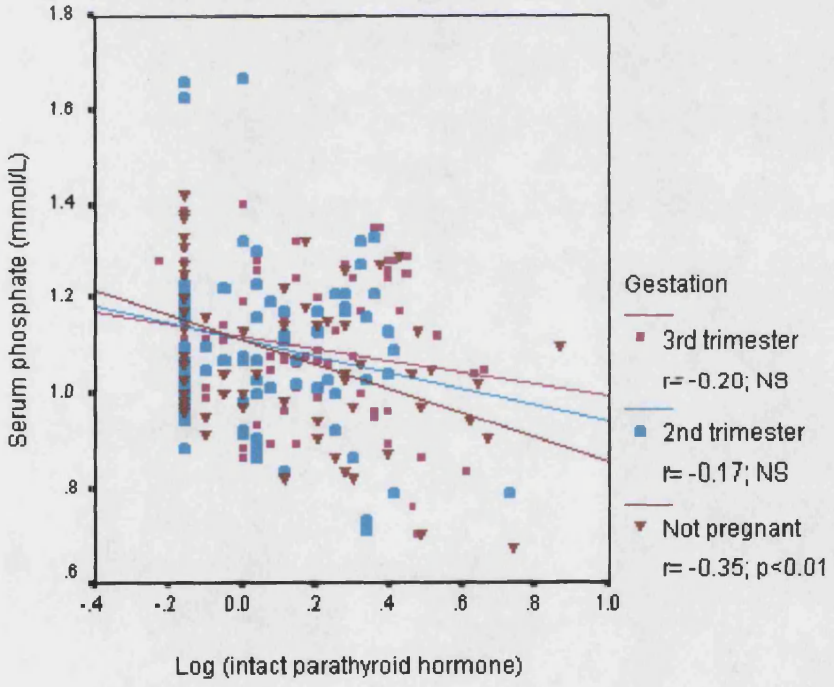


Not pregnant $\log_{10}[iPTH] = 2.24(0.67) - 1.73(0.54)iCa^{2+}$
2^{nd} trimester $\log_{10}[iPTH] = 0.93(0.46) - 0.72(0.38)Ca^{2+}$
3^{rd} trimester $\log_{10}[iPTH] = 2.36(0.45) - 1.86(0.37)iCa^{2+}$

Fig. 3.47 Correlations between log10[iPTH] and serum iCa^{2+} in normal (upper graph) and IDDM (lower graph) women at different gestations

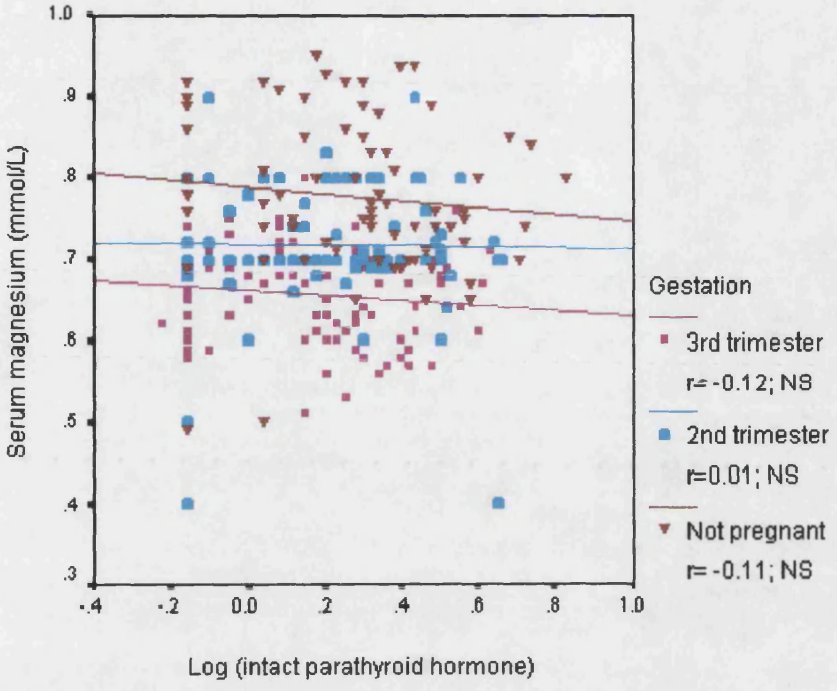


Not pregnant $[PO_4] = 1.32(0.03) - 0.27(0.08)\log_{10}[iPTH]$
2^{nd} trimester $[PO_4] = 1.13(0.02) - 0.26(0.06)\log_{10}[iPTH]$
3^{rd} trimester $[PO_4] = 1.15(0.03) + 0.11(0.08)\log_{10}[iPTH]$

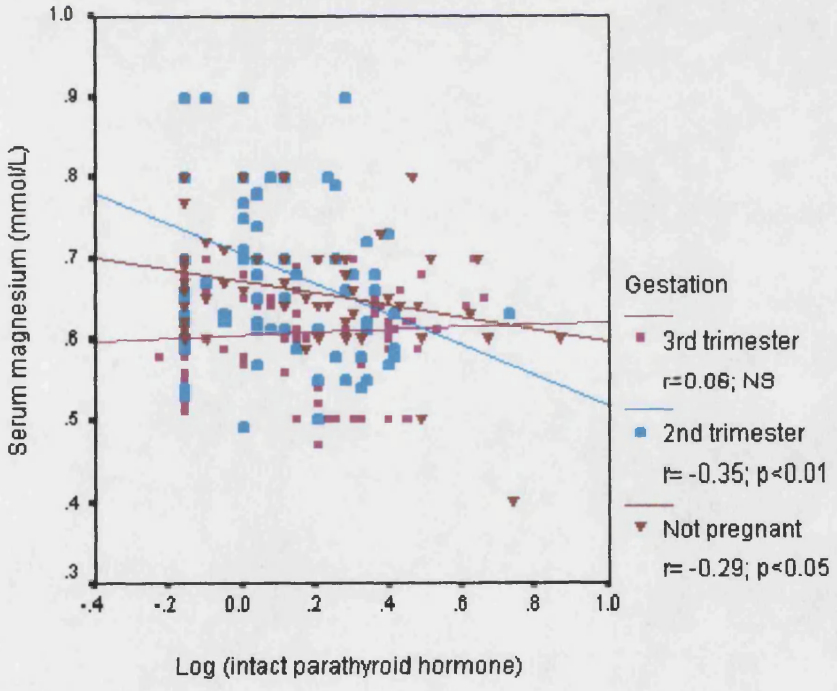


Not pregnant $[PO_4] = 1.11(0.02) - 0.26(0.08)\log_{10}[iPTH]$
2^{nd} trimester $[PO_4] = 1.11(0.02) - 0.17(0.11)\log_{10}[iPTH]$
3^{rd} trimester $[PO_4] = 1.12(0.02) - 0.12(0.07)\log_{10}[iPTH]$

Fig. 3.48 Correlations between $\log_{10}$[iPTH] and serum PO_4 in normal (upper graph) and IDDM (lower graph) women at different gestations



Not pregnant [Mg] = 0.79(0.02) – 0.11(0.04) $\log_{10}$[iPTH]
2nd trimester [Mg] = 0.71(0.01) – 0.01(0.04) $\log_{10}$[iPTH]
3rd trimester [Mg] = 0.66(0.01) – 0.01(0.03) $\log_{10}$[iPTH]



Not pregnant [Mg] = 0.67(0.01) – 0.20(0.03) $\log_{10}$[iPTH]
2nd trimester [Mg] = 0.71(0.01) – 0.19(0.06) $\log_{10}$[iPTH]
3rd trimester [Mg] = 0.61(0.01) – 0.04(0.03) $\log_{10}$[iPTH]

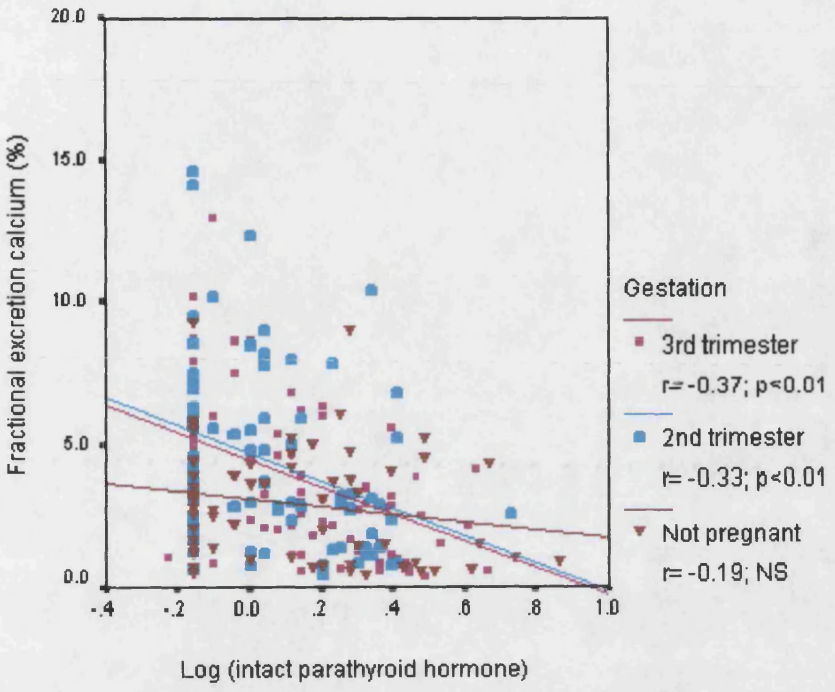
Fig. 3.49 Correlations between $\log_{10}$[iPTH] and serum Mg in normal (upper graph) and IDDM (lower graph) women at different gestations

Correlations between iPTH and urinary excretion rates

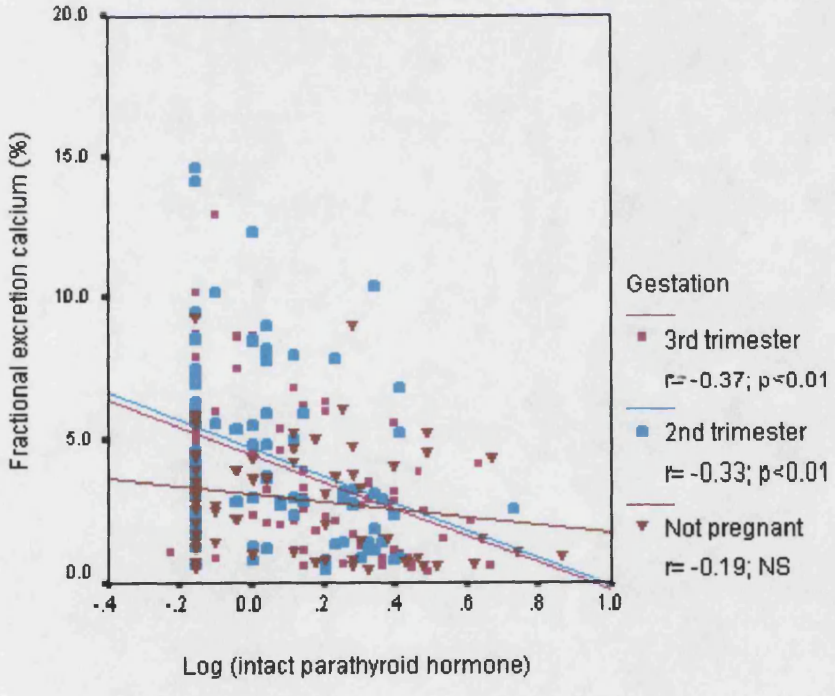
In the normal women there was a highly significant negative correlation between log_{10}[iPTH] concentration and the FECa: r=-0.22, $p<0.05$ at 21 weeks; r=-0.32, $p<0.01$ at 31 weeks; r=-0.24, $p<0.05$ outside pregnancy (fig.3.50). In the pregnant IDDM women the same highly significant correlations occurred at 21 weeks (r=-0.33, $p<0.01$) and 31 weeks (r=-0.37, $p<0.01$) but not outside pregnancy (r=-0.19, NS) (fig.3.50).

There was no correlation between log_{10}[iPTH] and the $FEPO_4$ at any gestation in either group of women.

Log_{10}[iPTH] and the FEMg were significantly negatively correlated in the normal women at 21 weeks (r=-0.31, $p<0.01$) and at 31 weeks
(r=-0.31, $p<0.01$) but not outside pregnancy (r=-0.15, NS) (fig.3.51). There were also significant negative correlations between these two variables in the IDDM women at 21 weeks (r=-0.31, $p<0.01$) and when not pregnant (r=-0.51, $p<0.001$) (fig. 3.51).

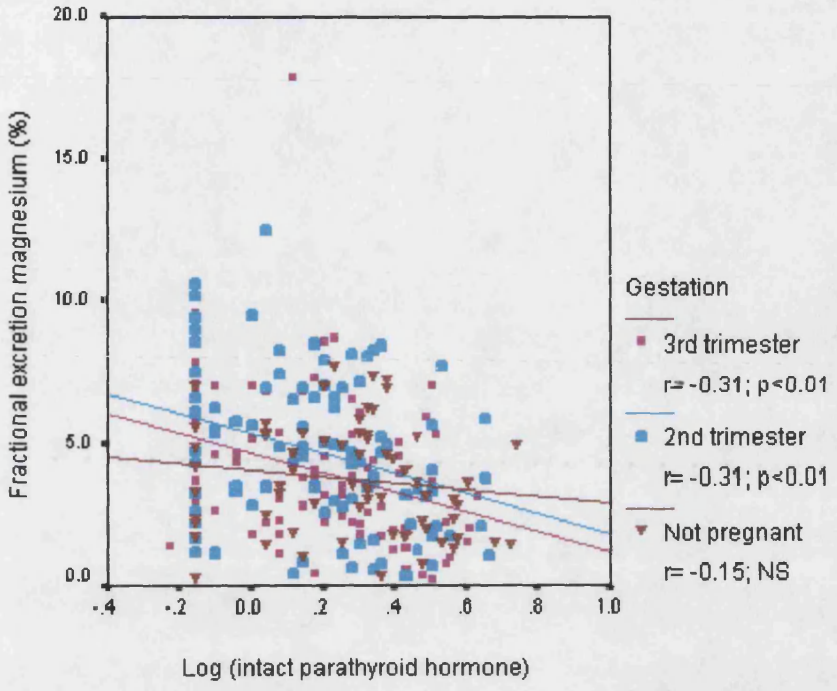


Not pregnant FECa = 3.95(0.45) – 2.49(1.24) $\log_{10}$[iPTH]
2nd trimester FECa = 4.56(0.42) – 3.17(1.43) $\log_{10}$[iPTH]
3rd trimester FECa = 5.13(0.45) – 4.80(1.52) $\log_{10}$[iPTH]

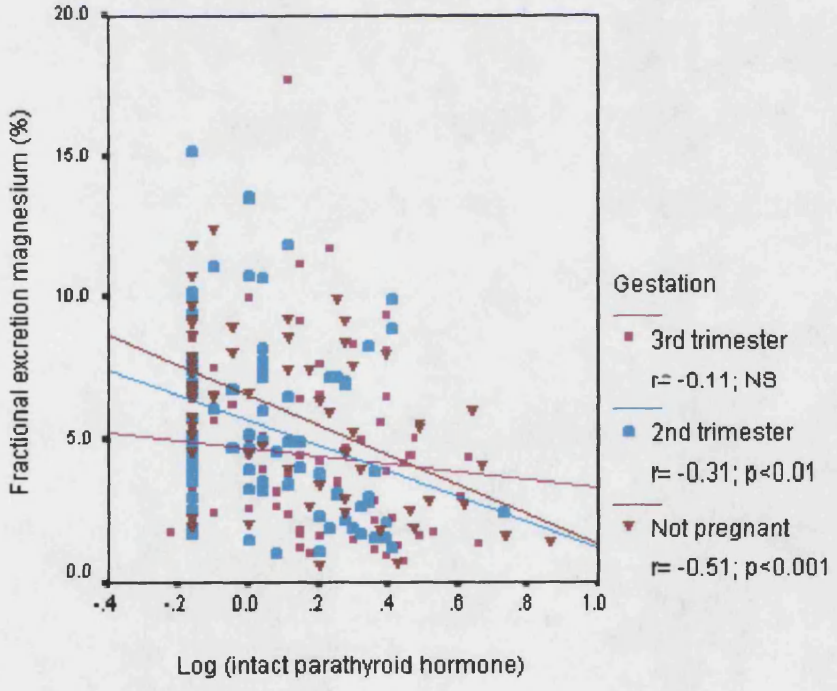


Not pregnant FECa = 3.11(0.27) – 1.37(0.89) $\log_{10}$[iPTH]
2nd trimester FECa = 4.77(0.34) – 4.91(1.57) $\log_{10}$[iPTH]
3rd trimester FECa = 4.54(0.35) – 4.80(1.31) $\log_{10}$[iPTH]

Fig. 3.50 Correlations between $\log_{10}$[iPTH] and FECa in normal (upper graph) and IDDM (lower graph) women at different gestations



Not pregnant FEMg = 4.07(0.35) – 1.15(1.00) $\log_{10}$[iPTH]
2nd trimester FEMg = 5.36(0.34) – 3.54(1.13) $\log_{10}$[iPTH]
3rd trimester FEMg = 4.66(0.34) – 3.48(1.16) $\log_{10}$[iPTH]



Not pregnant FEMg = 6.56(0.34) – 5.24(1.12) $\log_{10}$[iPTH]
2nd trimester FEMg = 5.66(0.34) – 4.49(1.55)) $\log_{10}$[iPTH]
3rd trimester FEMg = 4.70 (0.35) – 1.37(1.31) $\log_{10}$[iPTH]

Fig. 3.51 Correlations between $\log_{10}$[iPTH] and FEMg in normal (upper graph) and IDDM (lower graph) women at different gestations

I,25-DHCC CONCENTRATIONS

The original plan was to measure this in all the subjects but promised funding was withdrawn. However before this became apparent the laboratory did analyse a few fasting samples as shown in table 3.43. The same women were not analysed at the different gestations so comparisons between gestations could not be made. None of the IDDM women who developed PE had 1,25-DHCC measured.

Table 3.43 Fasting concentrations of 1,25-DHCC (pg/ml) in normal and IDDM women at 21 and 31 weeks gestation. Median (IQR).

	Normal women		IDDM women	
	21 weeks n=4	31 weeks n=7	21 weeks n=7	31 weeks n=7
1,25-DHCC	75.9(31.8-132.2)	84.7(75.8-128.0)	74.2(44.1-85.2)	75.7(58.6-87.5)

Although these figures show an 11% reduction in third trimester concentrations of 1,25-DHCC in the IDDM women when compared to the normal group this was not statistically significant.

3.4C Effects of acute calcium loading on PRC and PRS

1. Fasting values

In the normal women PRC rose significantly ($p<0.05$) with pregnancy, this rise was apparent by 21 weeks and there was no further significant rise between the 21 week data and the 31 week data (fig.3.52). When the 31 week data was subdivided into those receiving calcium or placebo it appeared that PRC was higher in the supplemented group but this was not statistically significant (table 3.44 and fig.3.54). PRS also increased significantly during pregnancy; the 21 week data were very significantly ($p<0.001$) higher than the data for outside pregnancy and there was also a rise ($p<0.05$) from 21 to 31 weeks gestation (fig.3.53). Calcium supplementation had no effect on PRS values (table 3.44 & fig.3.54).

Table 3.44 Fasting values of PRC (ng/ml/hr) and PRS (mcgAngI/ml) in normal and IDDM women at different gestations. Median (IQR)

		21 weeks	31 weeks placebo	31 weeks supplemented	Not pregnant
PRC	Normal	6.0(4.2-8.9)*	4.8(3.0-10.1)	9.6(6.0-17.4)*	2.7(0-6.3)
	IDDM	3.2(2.3-4.1)	4.5(2.1-7.5)	3.5(2.7-5.5)	2.7(0-4.9)
PRS	Normal	1.60(1.40-2.44)	2.70(1.48-3.21)	2.57(1.94-2.89)	0.68(0.43-1.00)
	IDDM	2.24(1.75-2.65)	2.81(2.17-3.04)	2.59(1.82-3.05)	0.55(0.37-1.30)

*$p<0.05$ comparing normal and IDDM

In the IDDM women there was a non-significant rise in PRC in pregnancy (table 3.44 & fig.3.52). PRS was significantly higher at 21 weeks when compared with the non-pregnant data ($p<0.001$) but there was no rise from 21 to 31 weeks (table 3.44 & fig.3.53). Calcium supplementation had no effect on PRC or PRS (fig.3.54).

Compared to normal women IDDM women had lower values of PRC in pregnancy at all gestations studied ($p<0.05$). PRS values were the same in both groups of women during and outside of pregnancy but the pattern of the pregnancy-induced rise was different in the IDDM women with no rise between the second and third trimesters (fig. 3.53).

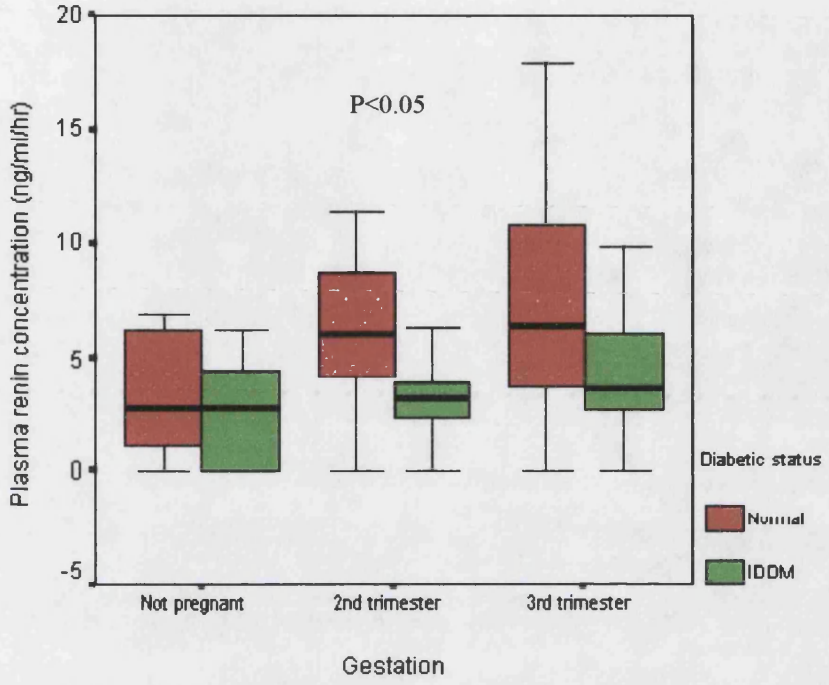


Fig. 3.52 Fasting PRC in normal and IDDM women by varying gestations of pregnancy. Values shown are median (IQR) (absolute range).

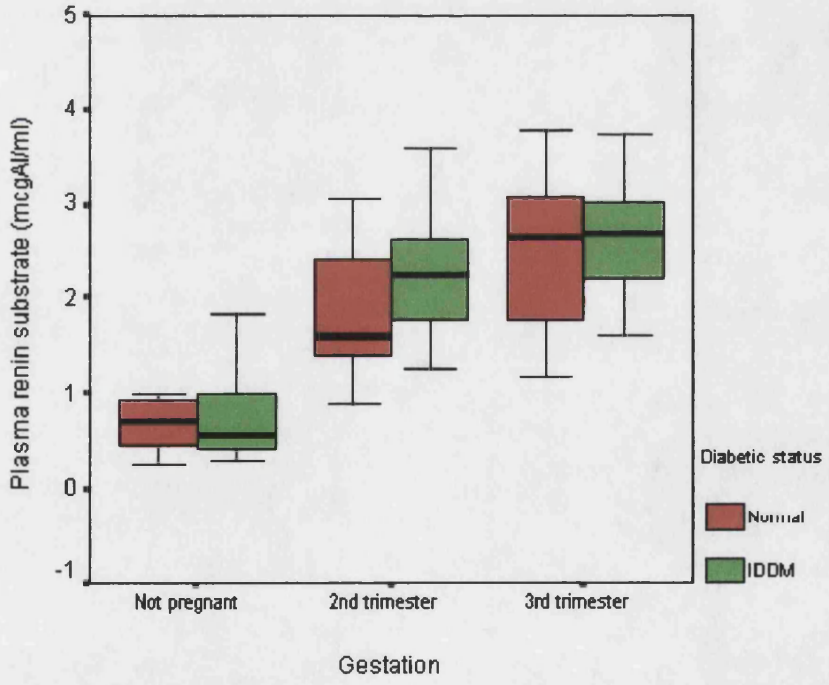
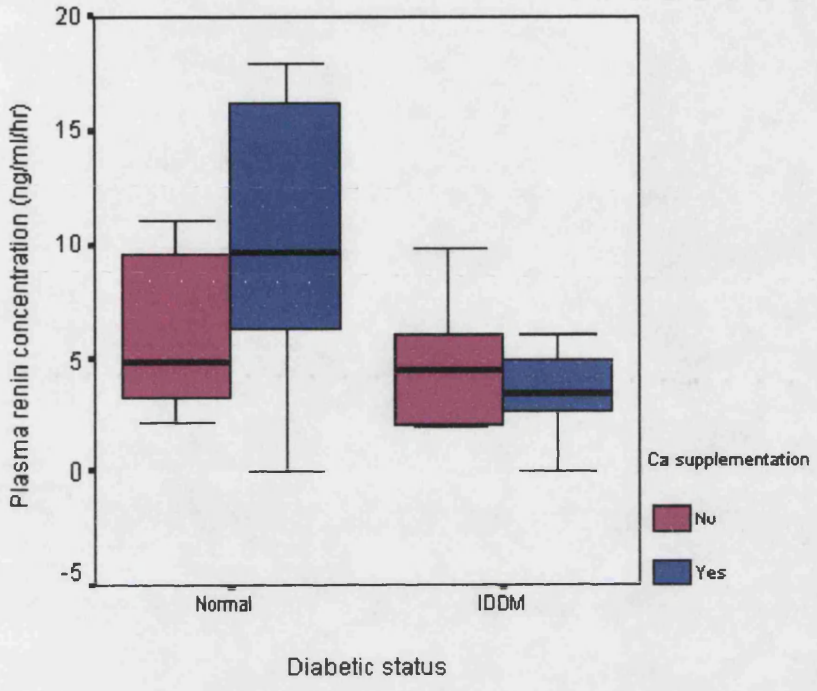


Fig. 3.53 Fasting PRS in normal and IDDM women by varying gestations of pregnancy. Values shown are median (IQR) (absolute range).



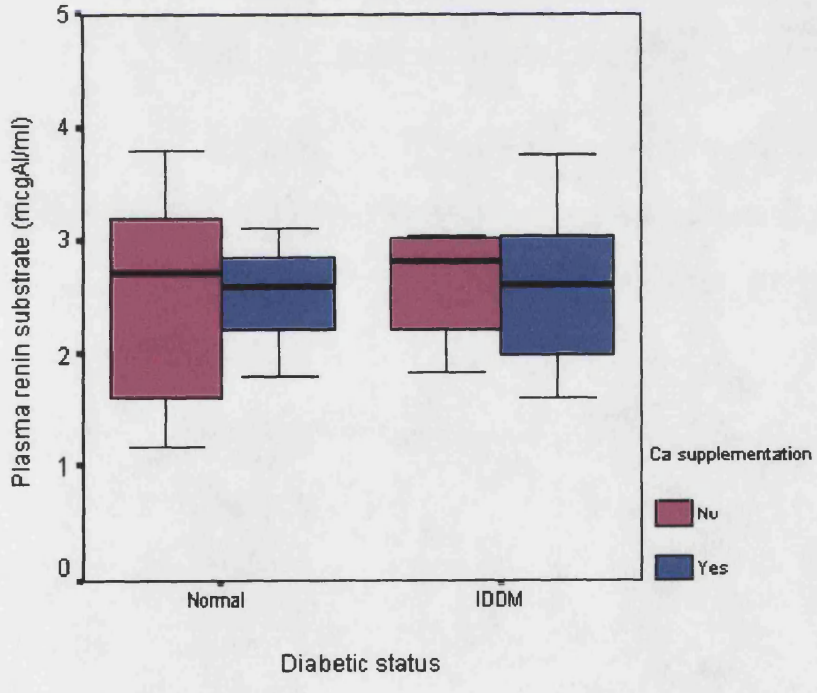


Fig. 3.54 The effect of calcium supplementation on PRC and PRS in normal and IDDM women in the third trimester of pregnancy. Values shown are median (IQR) (absolute range).

Effect of parity

Analysis of the second trimester data showed an effect of parity with the multiparous IDDM women having lower PRC than the normal multiparous women (p=0.014). This difference was not apparent in the primigravidae (p=0.2). By the third trimester all the IDDM women showed lower PRC than the normal women regardless of parity (table 3.45 & fig. 3.55). PRS was unaffected by parity (table 3.46).

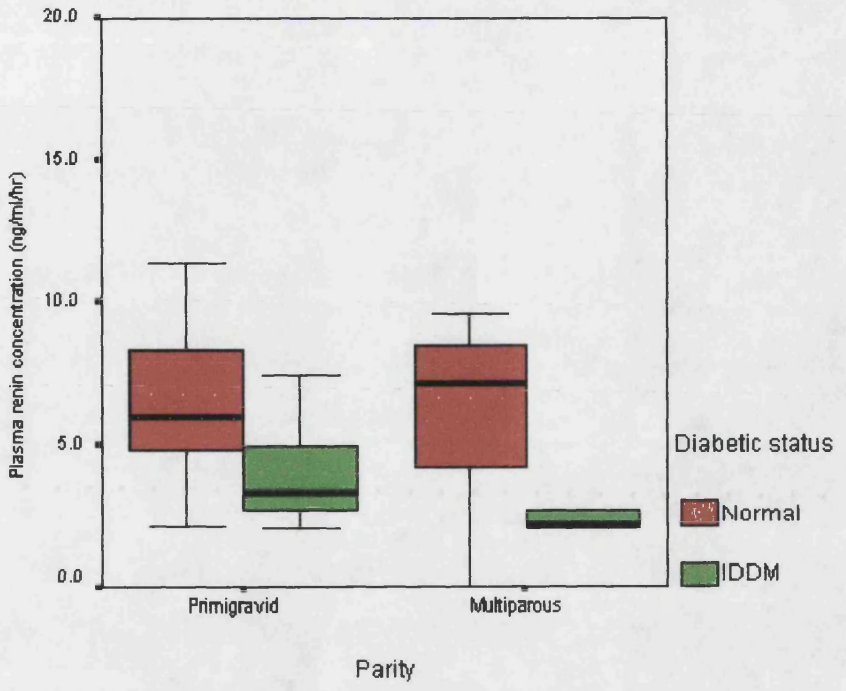
Table 3.45 Effect of parity on PRC (ng/ml/hr) in normal and IDDM women

	Second trimester		Third trimester	
	Normal	**IDDM**	**Normal**	**IDDM**
Primigravidae	6.0(3.8-9.6)	3.3(2.4-6.3)	8.3(5.7-17.6)	4.2(2.9-6.0)*
Multiparous	7.2(4.2-9.0)	2.2(1.1-3.5)*	5.4(2.6-10.0)	2.1(2.1-4.4)*

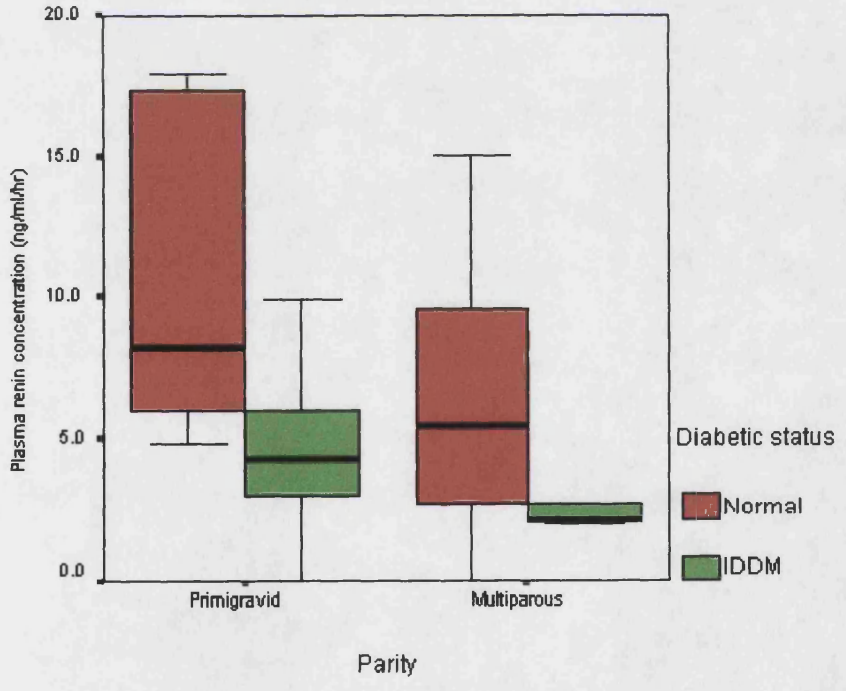
*$p<0.05$ comparing normal and IDDM

Table 3.46 Effect of parity on PRS (mcgAngI/ml) in normal and IDDM women

	Second trimester		Third trimester	
	Normal	**IDDM**	**Normal**	**IDDM**
Primigravidae	1.48(1.06-2.67)	2.28(1.97-2.67)	1.87(1.50-3.13)	2.75(2.16-3.04)
Multiparous	1.76(1.50-2.46)	1.81(1.29-3.56)	2.76(2.29-3.10)	2.57(2.17-4.40)



Second trimester



Third trimester

Fig.3.55 The effect of parity on PRC in normal and IDDM pregnancy in the second and third trimesters. Values shown are median (IQR) (absolute range).

PRC and PRS following the standard meal and oral Ca challenge

Although there appeared to be a rise in PRC in the normal women within one hour of the standard meal and oral calcium (table 3.48; fig. 3.56) this was not statistically significant. PRC in the IDDM women was also not affected by the standard meal (table 3.47; fig. 3.56). PRS in both the normal and the IDDM women was unaffected by the standard meal and oral calcium at any of the three periods studied (table 3.48; fig. 3.61).

Table 3.47 PRC (ng/ml/hr) and PRS (mcgAngI/ml) in normal and IDDM following a standard meal and calcium load at varying gestations. Median (IQR).

	21 weeks		31 weeks		Not pregnant	
	Normal	IDDM	Normal	IDDM	Normal	IDDM
Fasting PRC	6.0(4.2-8.9)	3.2(2.3-4.1)!	6.3(3.5-11.0)	3.6(2.6-6.0)*	2.7(0-6.3)	2.7(0-4.7)
PRS	1.6(1.4-2.4)	2.2(1.8-2.7)	2.6(1.8-3.1)	2.7(2.2-3.0)	0.7(0.4-1.0)	0.6(0.4-1.1)
1 hour PRC	7.8(5.1-9.6)	2.7(2.1-3.9)!	8.0(5.2-11.1)	4.5(2.9-7.0)!	3.6(0-6.6)	2.1(0-3.9)
PRS	2.0(1.4-2.4)	2.2(1.6-2.6)	2.1(1.9-3.1)	2.4(2.1-3.0)	0.5(0.3-1.8)	0.5(0.3-1.4)
2 hour PRC	6.5(4.6-8.6	2.6(2.1-4.1)!	8.7(5.4-11.4)	3.6(2.6-7.1)!	2.9(0-6.0)	2.4(0-5.42)
PRS	2.0(1.2-2.7)	2.3(2.0-2.7)	2.4(1.8-2.8)	2.2(2.0-2.6)	0.5(0.3-0.8)	0.5(0.4-1.5)
3 hour PRC	7.1(5.1-8.2)	3.5(2.8-4.7)!	6.3(4.5-9.6)	5.3(2.9-7.4)	3.9(0-6.6)	0(0-3.34)
PRS	2.1(1.4-2.7)	2.1(1.7-2.5)	2.4(1.8-2.7)	2.4(2.0-2.9)	0.6(0.4-1.4)	0.5(0.4-1.6)
4 hour PRC	6.9(4.8-9.1)	3.9(3.2-6.0)*	7.2(4.8-10.5)	3.3(2.6-7.2)*	2.9(0-4.8)	0(0-3.9)
PRS	2.0(1.3-2.4)	1.7(1.4-2.5)	2.2(1.7-2.5)	2.4(2.1-3.0)	0.7(0.5-1.2)	0.6(0.4-1.7)

*p<0.05 !p<0.01 comparing normal and IDDM

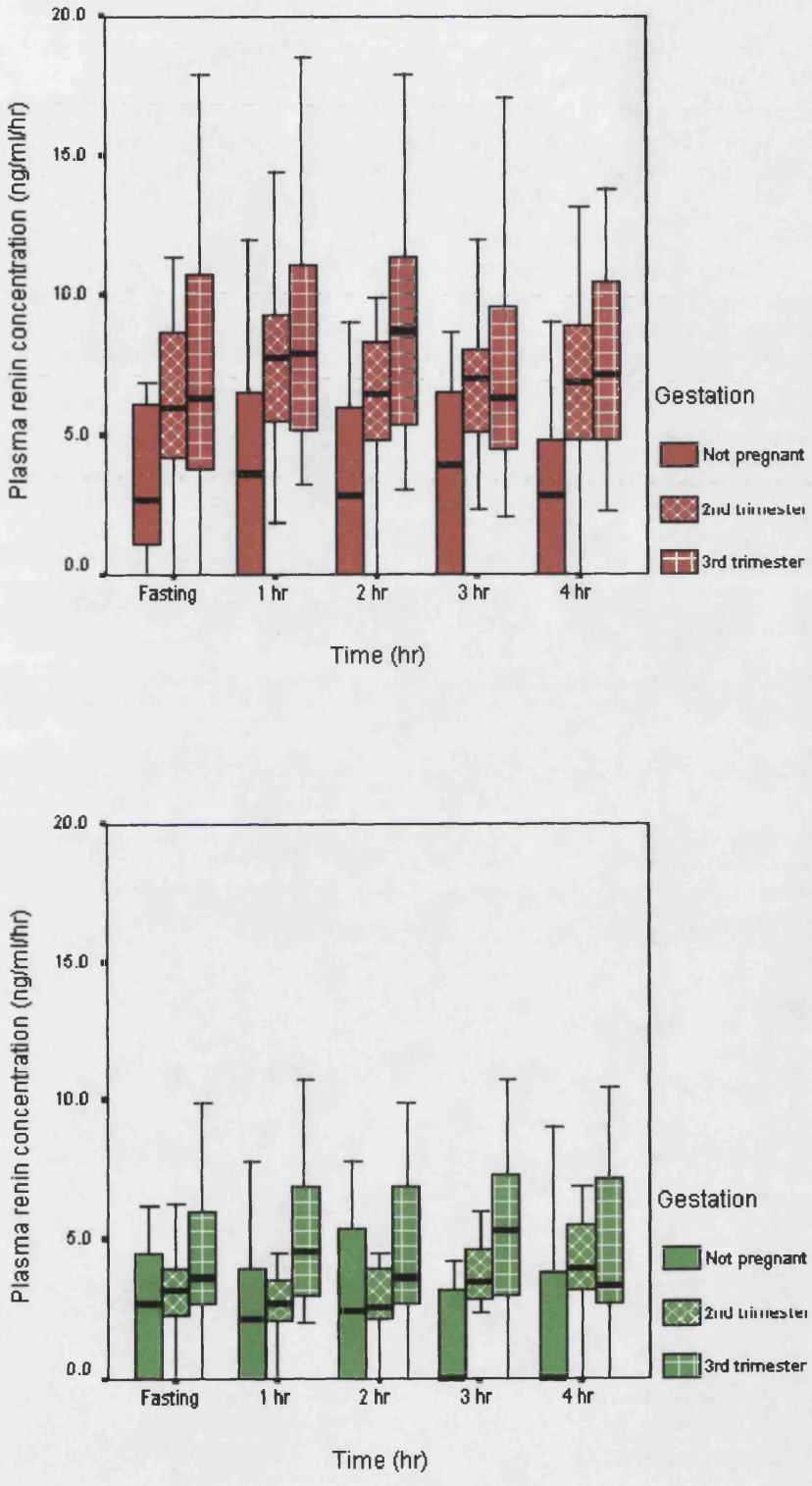
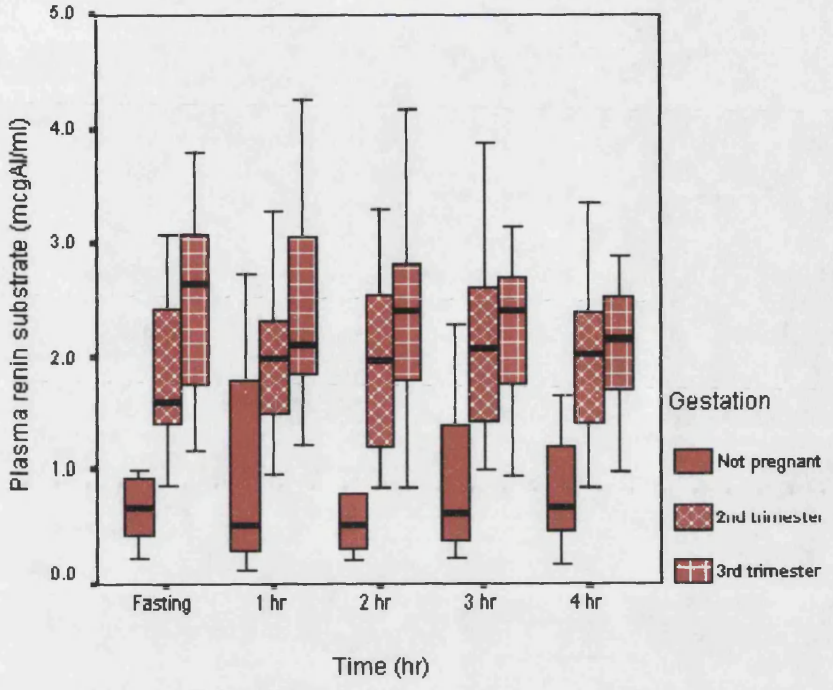


Fig.3.56 PRC in normal □ (upper graph) and IDDM □ (lower graph) women before and after the standard meal and oral Ca load at the three study gestations.



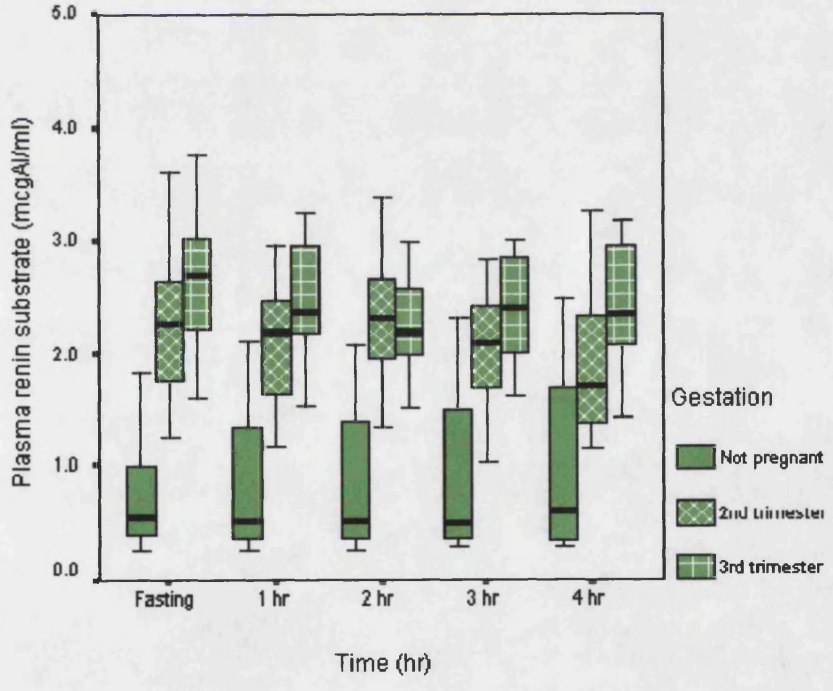


Fig.3.57 PRS in normal □ (upper graph) and IDDM □ (lower graph) women before and after the standard meal and oral Ca load at the three study gestations.

PRC and PRS results for the IDDM women who developed PE are shown in table 3.48. Of particular note are the high values found in D5 at 31 weeks gestation, approximately one week prior to her admission with clinically apparent PE.

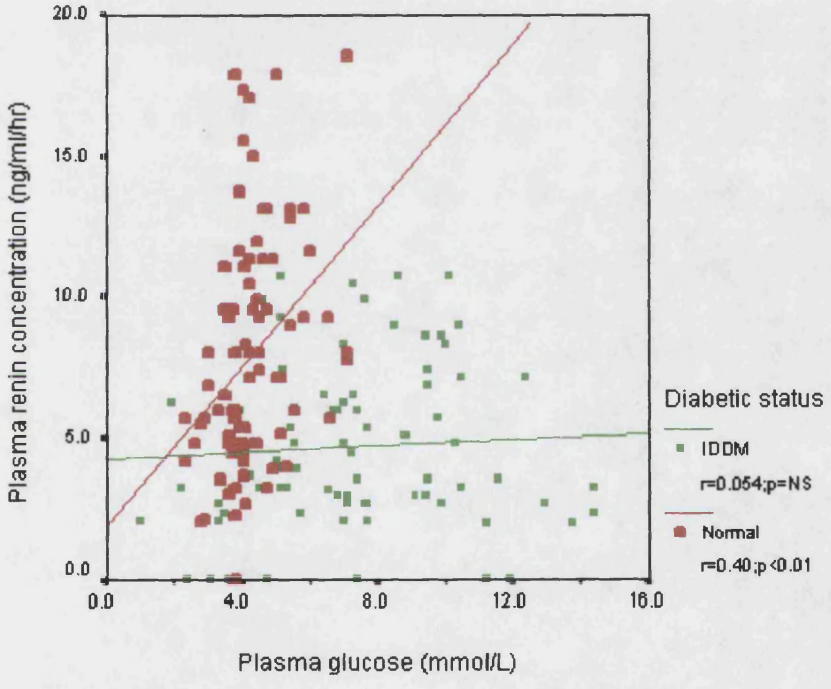
Table 3.48 PRC(ng/ml/hr) & PRS(mcgAngI/ml) in IDDM women who developed PE

	D3			D5		D10		
	21wk	**31wk**	**NP**	**21 wk**	**31wk**	**21wk**	**31wk**	**NP**
Fasting PRC	2.1	0	0	0	12.6	4.8	0	0
PRS	2.34	2.28	0.65	3.62	5.43	1.67	1.75	1.08
1 hour PRC	0	0	0	2.4	9.6	4.8	0	2.1
PRS	2.93	1.95	0.81	2.84	4.78	1.55	1.43	0.67
2 hour PRC	2.1	0	0	-	9.0	6.6	0	2.1
PRS	2.77	1.93	0.89	-	3.62	1.66	1.53	1.37
3 hour PRC	0	0	0	2.7	13.2	8.1	0	2.7
PRS	1.81	2.30	0.88	4.29	5.26	1.69	1.18	1.30
4 hour PRC	2.4	0	0	-	10.5	4.8	0	2.7
PRS	3.27	1.76	1.21	-	4.45	1.83	1.03	1.28

3.4D Correlations between the RAS and other variables

Glucose

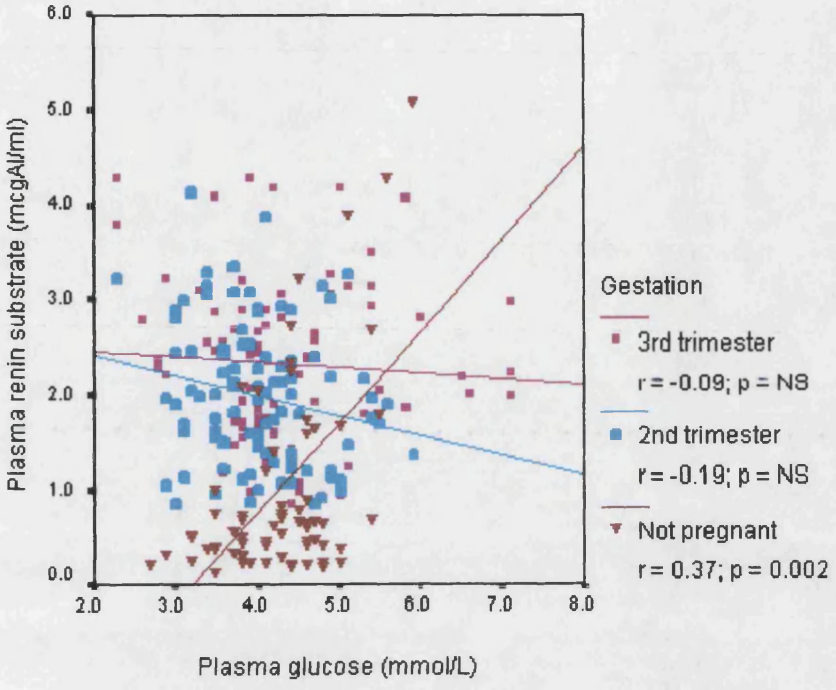
There was no significant correlation between plasma glucose and PRC in either group of women outside pregnancy (r = -0.14,NS for normal women; r = 0.07,NS for IDDM women). At 21 weeks there was also no correlation (r = 0.02,NS for normal women; r = –0.11,NS for IDDM women). At 31 weeks there was a very significant positive correlation between PRC and plasma glucose in the normal women (r=0.4; p<0.01) although not in the IDDM subjects (r=0.05; NS) (fig. 3.58).



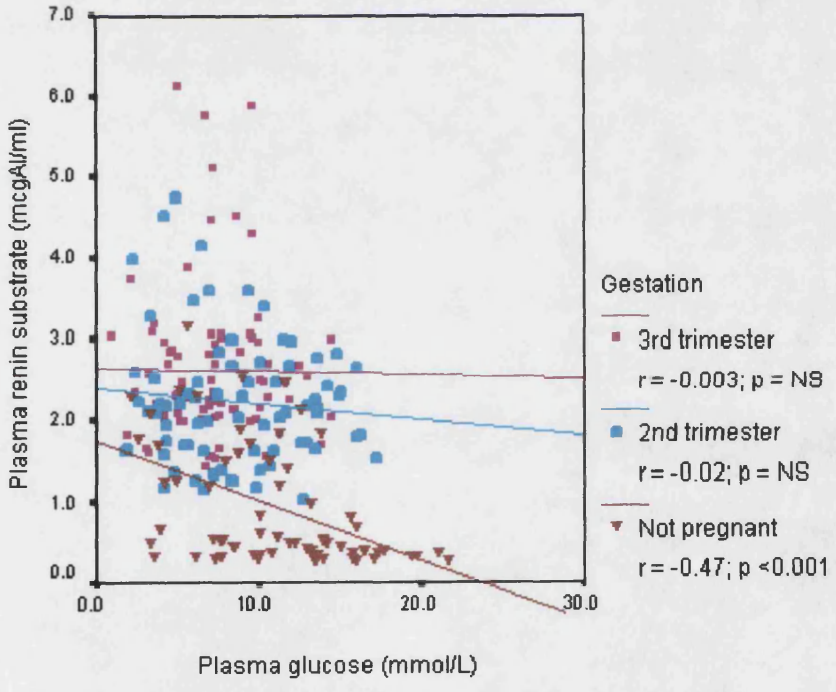
Normal[PRC] = 1.94(1.87) + 1.42(0.43)[glu]
IDDM[PRC] = 4.29(0.85) + 0.05(0.12)[glu]

Fig. 3.58 Correlation between plasma glucose and PRC in the third trimester

PRS did not correlate with glucose during pregnancy in either group of women. In the non-pregnant subjects there was a significant positive correlation between these variables (r=0.57;p<0.01) in the normal women but a negative correlation (r=-0.47; p<0.001) in the diabetic women (fig. 3.59).



Not pregnant PRS = -3.09(0.75) + 0.96(0.17)glucose
2nd trimester PRS = 2.85(0.43) – 0.21(0.10)glucose
3rd trimester PRS = 2.60(0.39) – 0.16(0.01)glucose



Not pregnant PRS = 1.73(0.21) – 0.20(0.02)glucose
2nd trimester PRS = 2.41(0.20) – 0.05(0.02)glucose
3rd trimester PRS = 2.65(0.28) – 0.01(0.04)glucose

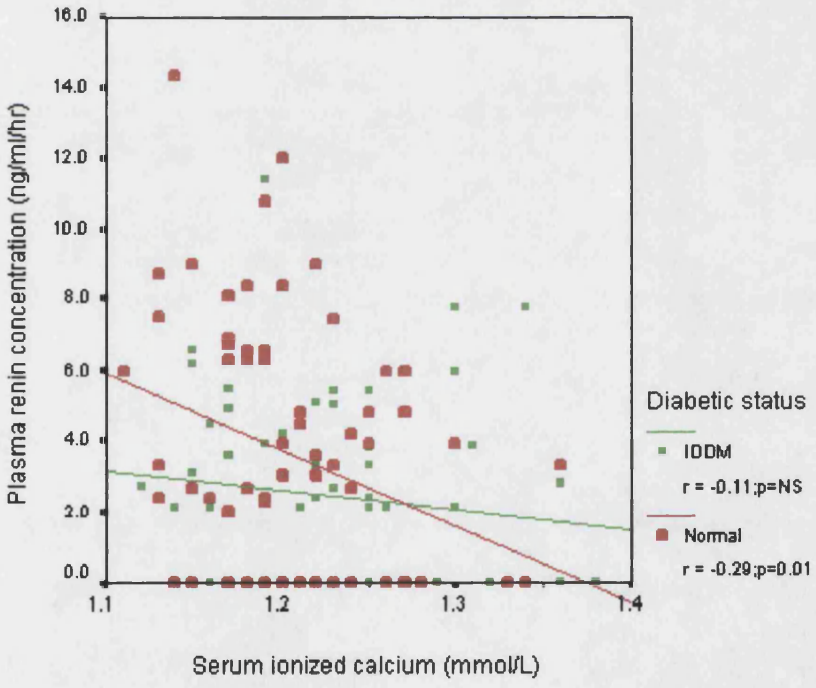
Fig. 3.59 Correlations between plasma glucose and PRS in normal (upper graph) and IDDM (lower graph) women at different gestations

Sodium and potassium

Plasma sodium and potassium did not correlate with either PRC or PRS in either the normal or the IDDM subjects at any gestation.

Ionised calcium

There was a significant (p=0.01) negative correlation between serum iCa^{2+} and PRC in the non-pregnant normal women (r = -0.29) but this association did not persist into pregnancy (r=-0.14;NS) at 21 weeks, (r= -0.19;NS) at 31 weeks. There was no similar correlation in the non-pregnant IDDM women (r= -0.11;NS) (fig.3.60). There were also no significant correlations between PRC and serum iCa^{2} in the pregnant IDDM women. Serum iCa^{2+} did not correlate with PRS in either group of women at any gestation.



Normal[PRC] = 29.69(9.36)-21.58(7.74)[iCa^{2+}]
IDDM[PRC] = 8.95(7.01)-5.30(5.70)[iCa^{2+}]

Fig. 3.60 Correlation between serum iCa^{2+} and PRC in the non-pregnant women

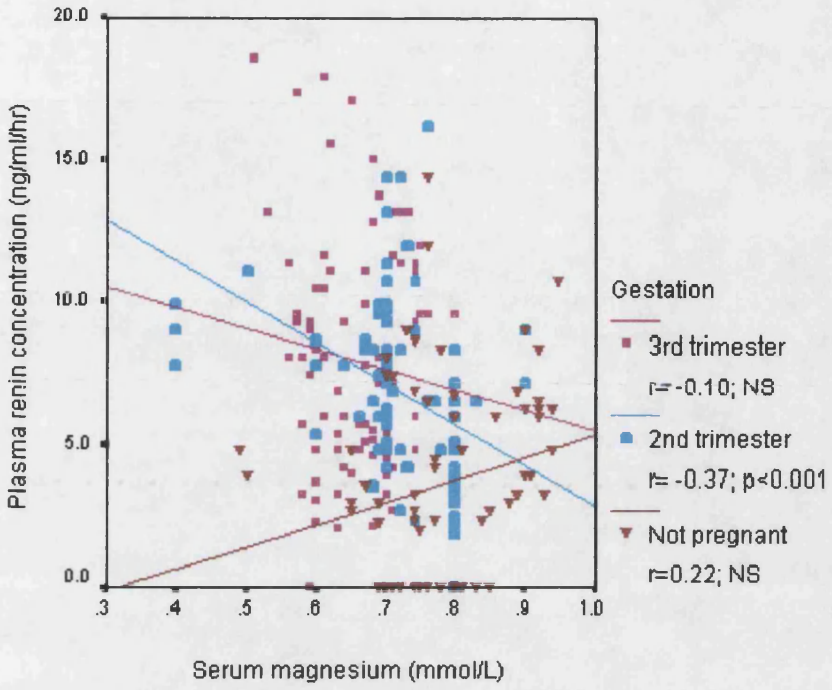
Magnesium

There were also significant correlations between serum Mg and PRC but these were not consistent (fig. 3.61). In the normal women at 21 weeks there was a negative correlation ($r=-0.37;p<0.001$) but at 31 weeks no correlation ($r=-0.10$;NS). Outside pregnancy there was a positive correlation which just failed to reach significance ($r=0.22;p=0.07$). In the IDDM women there was no association outside pregnancy ($r=-0.09$;NS) or at 21 weeks ($r=0.13$;NS) but at 31 weeks there was a significant negative correlation ($r= -0.26$; $p<0.05$). Serum Mg was not correlated with PRS.

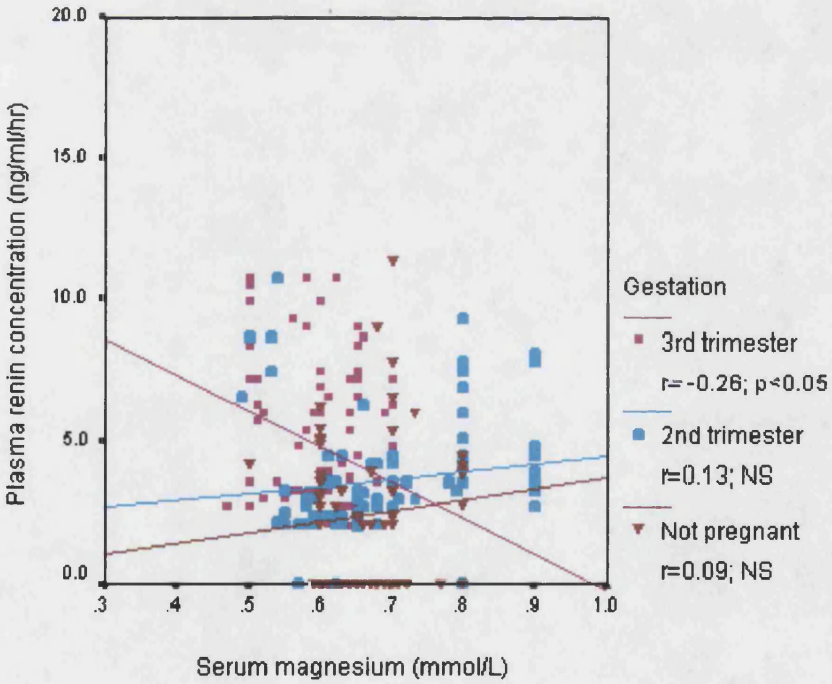
Intact PTH

There was a significant negative correlation ($r= -0.3;p=0.007$) between PRC and iPTH in the IDDM subjects at 21 weeks gestation but not at any other gestation and there was no correlation between PRC and iPTH in the normal subjects at any gestation. PRS did not correlate with iPTH outside pregnancy but during pregnancy there was a significant negative correlation between the two hormones: for the normal women at 21 weeks $r= 0.25$; $p<0.05$ and at 31 weeks $r= -0.28$; $p<0.01$; for the IDDM women at 21 weeks $r= -0.24$; $p<0.05$. At 31 weeks in the IDDM group there was no correlation (fig. 3.62).

When the intercepts on the y axis of fig. 3.62 were compared by t-test there was a significant ($p<0.001$) difference between the pregnant, both 2nd and 3rd trimesters, and the non-pregnant data for both groups of women. Diabetic status did not significantly affect the values of the intercepts. In the normal women although the slopes of the 2nd and 3rd trimester graphs looked different to the non-pregnant slope this was not confirmed by t-test. The slopes of the IDDM graph were not compared as only one of these graphs illustrated a significant correlation.

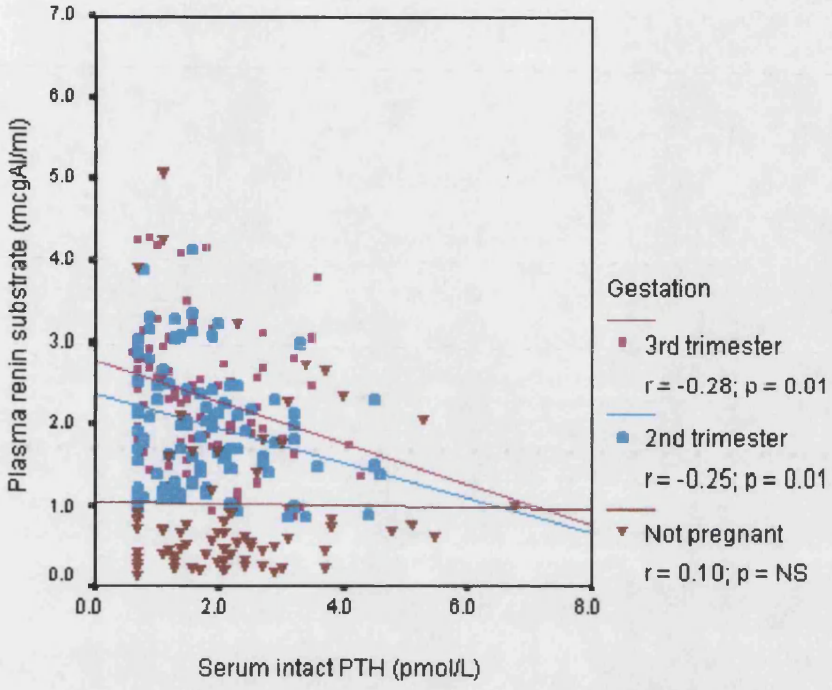


Not pregnant PRC = -2.51(3.36) + 7.86(4.29)Mg
2nd trimester PRC = - 17.27(2.68) – 14.38(3.40)Mg
3rd trimester PRC = 12.73(5.00) – 7.19(7.53)Mg

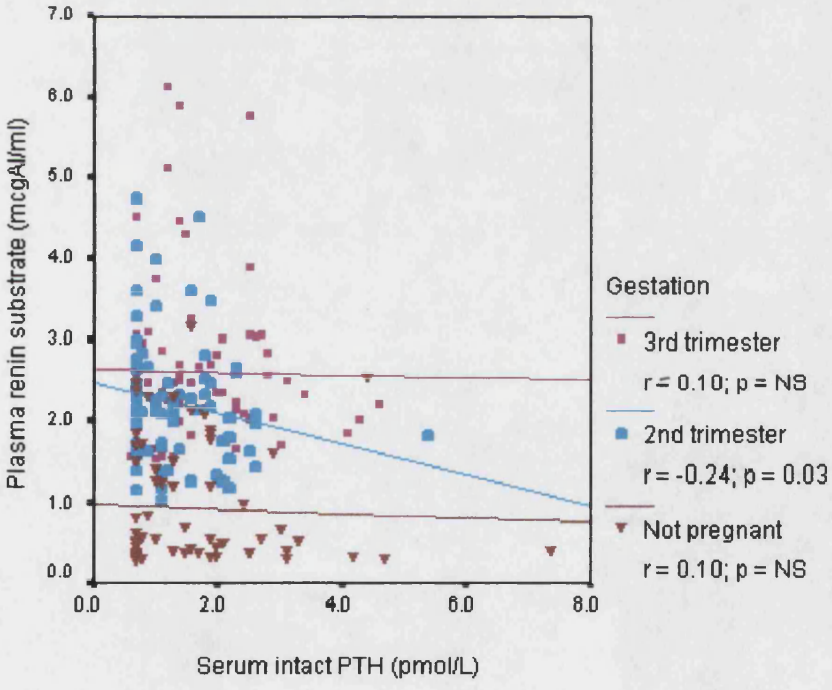


Not pregnant PRC = -0.20(3.68) + 3.78(5.49)Mg
2nd trimester PRC = 1.94(1.52) + 2.54(2.17)Mg
3rd trimester PRC = 12.37(3.23) – 12.62(5.28)Mg

Fig. 3.61 Correlations between serum magnesium and PRC in normal (upper graph) and IDDM (lower graph) women at different gestations



Not pregnant [PRS] = 1.06(0.27) – 0.01(0.10)[iPTH]
2nd trimester [PRS] = 2.38(0.15) – 0.22(0.08)[iPTH]
3rd trimester [PRS] = 2.78(0.18) – 0.25(0.09)[iPTH]



Not pregnant [PRS] = 0.97(0.16) – 0.03(0.08)[iPTH]
2nd trimester [PRS] = 2.48(0.17) – 0.19(0.11)[iPTH]
3rd trimester [PRS] = 2.64(0.20) – 0.02(0.11)[iPTH]

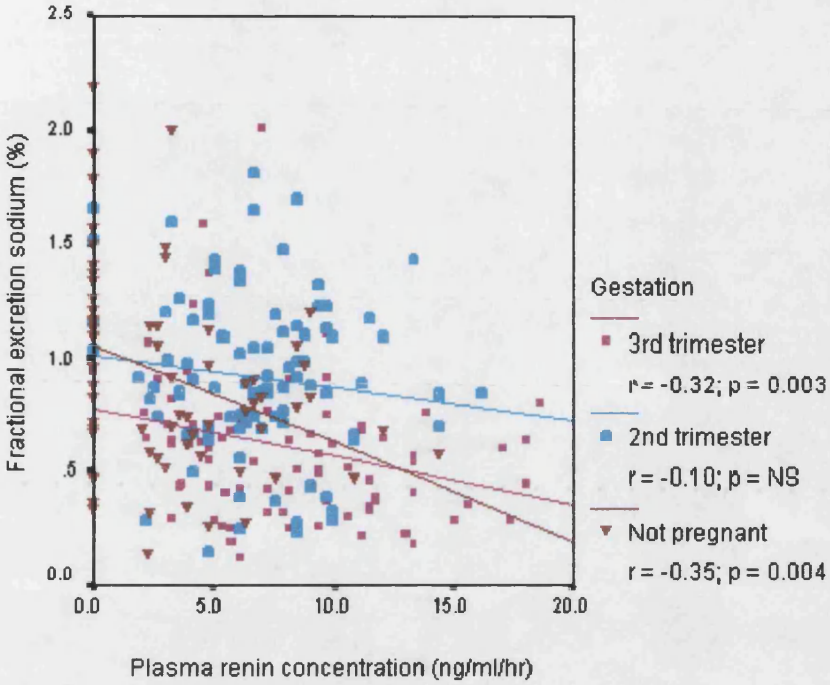
Fig. 3.62 Correlations between PRS and serum iPTH in normal (upper graph) and IDDM (lower graph) women at different gestations

Fractional excretion of sodium and potassium

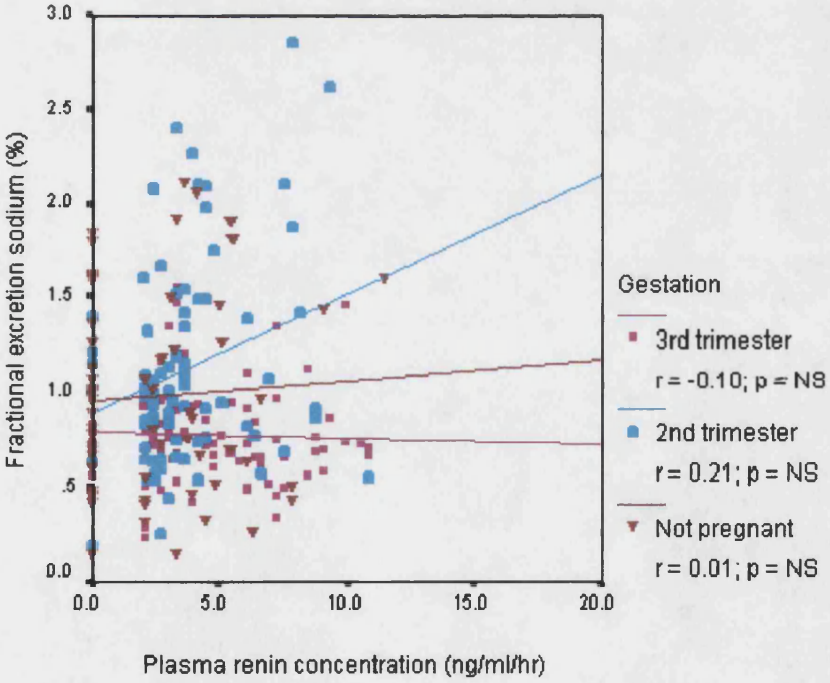
In the normal women there was a significant, negative correlation ($r= -0.35; p<0.01$) between PRC and FENa; this was also apparent at 31 weeks gestation ($r= -0.32; p<0.01$) but was lost at 21 weeks gestation ($r= -0.96$;NS). In the IDDM women there was no correlation between these two variables at any gestation (fig. 3.63). PRS did not correlate with FENa.

FEK in the normal women did not correlate with either PRC or PRS at any gestation. In the pregnant IDDM women there was a positive correlation between PRC and FEK ($r=0.27; p<0.05$ at 21 weeks and $r=0.41; p<0.001$ at 31 weeks). This correlation was not apparent in the non-pregnant IDDM women.

PRS correlated negatively with the FEK in the non-pregnant IDDM women ($r= -0.32; p<0.01$) but there were no other significant correlations between these variables.



Not pregnant FENa = 1.06(0.07) – 0.04(0.01)[PRC]
2nd trimester FENa = 1.02(0.09) – 0.01(0.01)[PRC]
3rd trimester FENa = 0.78(0.07) – 0.02(0.01)[PRC]



Not pregnant FENa = 0.95(0.09) = 0.01(0.02)[PRC]
2nd trimester FENa = 0.89(0.11) = 0.62(0.03)[PRC]
3rd trimester FENa = 0.79(0.05) – 0.01(0.01)[PRC]

Fig. 3.63 Correlation between PRC and FENa in normal (upper graph) and IDDM (lower graph) women at different gestations

DISCUSSION

This study has found interesting differences between normal and IDDM pregnancy both in terms of the renal handling of specific substances and the intact parathyroid hormone response to oral calcium loading. Some of these differences confirm previous work whilst others are new findings. They will be discussed below and an attempt will be made to explain them.

The findings about the RAS in IDDM pregnancy confirm differences between these and normal pregnancies found by earlier workers; to our knowledge the RAS has not been studied prospectively in the same IDDM women during and after pregnancy. The finding of very high PRC and PRS just prior to the onset of PE in one diabetic woman has raised the possibility that altered RAS activity in IDDM pregnancy may partially account for the increased incidence of PE in these pregnancies. Possible differences in the RAS of primigravid and multigravid IDDM women were also found which have not previously been reported.

There were numerous flaws in this study some of which could have been prevented by better planning; however many were as a result of dealing with human volunteers who inevitably form a more heterogenous group than laboratory rats! These problems will be discussed in greater detail below. Finally this discussion will end by suggesting future areas of research which could build on these preliminary findings whilst hopefully avoiding many of the pitfalls into which studies in this thesis fell.

4.1 Study design

This study was inevitably restricted by the fact that it was carried out mainly by one investigator with finite time. It was also limited by the number of diabetic pregnancies which occurred in the unit during the period of research. As the studies required time and prolonged commitment from the volunteers, not all pregnant IDDM women were suitable and willing for recruitment.

Study protocol

Previous work done in our department (Bisson, 1992) had made a serendipidous discovery of differences between iPTH levels in normal (n=18) and IDDM (n=16) pregnant volunteers. At the time (1993-4) when this study was designed there was great interest in the use of calcium supplementation to prevent the development of PE. Initial studies on this had shown significant changes in BP produced by calcium supplementation in small groups of women. For example Belizan reported results from Guatemala in 1983 in which 14 women received placebo and 22 women received either 1g or 2g calcium supplementation per day (Belizan *et al,* 1983[a]). Knight & Keith (1992) showed a reduction in DBP in 10 American women with PIH who received 1g calcium compared with 10 women with PIH on placebo. The problem with these initial studies is that "treatment" of a very small number of heterogenous subjects will always produce suspect results as so many other factors besides the "treatment" could be responsible for the observed differences. However further work with larger numbers of non-European women did seem to support the hypothesis that calcium supplementation reduced the incidence of hypertensive complications of pregnancy (Villar et al, 1987 [1.5g calcium, 52 women]; Lopez-Jaramillo et al, 1989 [2g calcium, 106 women]; Belizan et al, 1991 [2g calcium, 1194 women]).

Other studies have shown an association between hyperparathyroidism and hypertension as reviewed in the introductory section of this thesis (section 1.2H, p26). In summary this link was first described in 1957 by Hellstrom et al, who found that 70% of patients with hyperparathyroidism were hypertensive at some stage of the disease. Initially this association was though to be due to impaired renal function in patients with hyperparathyroidism. A study in 1981 found that in 42 hyperparathyroid patients with hypertension only 4 had raised serum creatinine levels. As increases in serum creatinine only occur once there has been a significant fall in glomerular filtration rate in was unfortunate that this study did not use a more sensitive marker of renal function (Lafferty, 1981). Subsequently a Dutch group looked at *intact* PTH and showed that this is increased in young (20-39 year old) primary hypertensive subjects (Grobbee et al, 1988). McCarron et al (1980) suggested that hyperparathyroidism might be a physiological response to a urinary calcium leak associated with essential hypertension.

Whilst a large, multicentre, prospective, randomized controlled trial was obviously needed to establish whether or not calcium was of use in preventing PE it was felt that it would be useful to study calcium handling and iPTH, comparing normal pregnant women with IDDM pregnant women who would be at increased risk of developing PE (Garner *et al*, 1990). The study was designed to be prospective and it was felt from the outset that it was important to follow as many of the women as possible into the post-partum period and beyond so that each woman could act as her own control. The calcium load experiments were designed to look at the short-term changes in serum electrolytes and *intact* PTH ,and urinary electrolyte excretion rates induced by oral calcium whilst the double-blind randomized allocation to either calcium supplements or placebo would look at the longer term effects of oral calcium. Whilst not looking primarily for an effect of calcium supplementation on BP it was thought prudent to record BP on a regular basis throughout the time the women received therapeutic intervention.

1.5g of calcium was chosen as the supplementary dose having considered several other trials which had used amounts which varied from 1g to 2g (Belizan *et al,* 1983[a]; Villar *et al,* 1987; Lopez-Jaramillo *et al,* 1989; Belizan *et al,* 1991; Knight & Keith, 1992). It was also a dose in line with the recommended intake for pregnancy of 1250mg calcium daily (Committee On Medical Aspects of food policy, 1991).

1g of elemental calcium was chosen for the oral calcium tolerance load in accordance with work described by Broadus, Dominguez and Bartter (1978) on non-pregnant women and subsequent work by Kent, Price, Gutteridge, Allen, Blakeman, Bhagat, St John, Barnes, Smith & Evans (1991) on pregnant women. Broadus kept his participants as in-patients which allowed their calcium intake prior to the oral load to be strictly controlled, this was not felt to be practical for this study. He measured urinary calcium excretion for 6 hours after oral calcium and found that it increased within 2 hours, plateaued for 2 hours and then decreased. During the whole of this 6 hour period his patients were not allowed other food. Kent *et al* (1991) described a study period of four hours after oral calcium. This was long enough to show rises in serum iCa^{2+} and UCE and a fall in iPTH but not long enough to show a return to normal values. It was felt that, for this thesis, reducing the test to four hours made the test more acceptable to pregnant women whilst not losing much information. It also

avoided the need for intravenous dextrose and insulin in the IDDM group which again made the test more acceptable to pregnant women.

PRC and PRS were measured in the same women as increasing evidence of an interaction between the RAS and the calcium/PTH system is being amassed in non-diabetic subjects both outside and during pregnancy. Subjects with essential hypertension and a low-renin profile respond well to calcium channel blockers while those with a high-renin profile are relatively insensitive to this class of drugs (Resnick, Nicholson & Laragh, 1986). Elderly hypertensive patients with low plasma renin and high levels of aldosterone have been shown to have low serum iCa2+ and high iPTH levels (Zozaya & Viloria, 1997). Infusion of magnesium sulphate in pregnant women with PE has also been shown to reduce PRA (Sipes, Weiner, Gellhaus & Goodspeed, 1989). Also although virtually nothing is known about the RAS in IDDM pregnancy, it is known to be disordered in non-pregnant diabetic subjects (see section 1.3H, p48).

Numbers and characteristics of recruited women

Our hospital looked after approximately 16 IDDM pregnant women per year, excluding those gestational diabetic women who required insulin treatment. We felt that we had a realistic chance of recruiting 20 reliable IDDM women who would be prepared to embark on a prospective study in the time available and as this number was of the same order as the studies described above we hoped to be able to detect significant differences between them and normal controls in calcium metabolism. There were of course potentially more normal pregnant women who could be enrolled into the study. However as this was quite a complicated study involving three complete morning sessions in the laboratory, 24 hour urine collections, therapeutic intervention and numerous visits for BP recording in the end it proved quite difficult to recruit even 20 normal women within the allocated time. Only one normal woman who was not a member of staff at the hospital agreed to take part. Inevitably this meant that the control group was significantly different from the IDDM group in terms of occupation. However as all 40 women were Caucasian and lived in the same area it seemed unlikely that the occupation bias in the control group would be large. If anything the high proportion of doctors, nurses and midwives in the control group should have helped increase the accurate collection of tests such as the 24 hour

collection of urine. It also probably increased their motivation towards the study which was high, judging by both their compliance with calcium or placebo supplementation, and their willingness to return for repeated studies. The IDDM group were also well motivated and demonstrated high compliance, reflected in their strict adherence to a tight diabetic regime during the pregnancy. Diabetic control was significantly less good post-partum (a classical feature) and it is likely that compliance with supplementation would also have been less good outside pregnancy.

More IDDM women (14 out of 20) in the study were primigravid, compared with 9 of 20 in the control group. Ideally it would have been best to recruit only primigravid women or else a larger number of women of mixed parity so that the women could have been subdivided into primigravid and multigravid patients and analysed separately. The subgroup of 6 multiparous IDDM patients was really too small a group for meaningful statistical analysis. Nevertheless the results were analysed by parity as indicated in the results chapters in an attempt to reduce any bias caused by the unequal parity distributions in the two groups of women. Predictably the multiparous patients were significantly older than the primigravid patients in both groups but only by a few years. Although renal function deteriorates with age one would not have expected this small age difference to produce a detectable effect and this was confirmed by the lack of parity difference in baseline serum biochemistry, creatinine clearance and fractional excretion rates in both normal and IDDM women. The parity differences which were found are discussed in greater detail below but must be regarded with caution because of the small numbers involved.

IDDM women with hypertension or nephropathy, defined as an abnormal urinary albumin:creatinine ratio, were not recruited for this thesis. This sub-set of IDDM women is known to be at particularly high risk of developing PE, probably because of pre-existing vascular endothelial dysfunction. As this thesis was looking specifically at disorders in either calcium homeostasis or the RAS it was decided that the inclusion of a complicated sub-set of women would only confuse the issues under investigation.

Timing of the post-pregnancy studies

Calcium metabolism is affected by lactation. Kent *et al* (1991) found that lactating women had very significantly reduced UCE following oral calcium loading compared

with either pregnant or non-pregnant women. There is no evidence that serum iCa^{2+} concentrations are affected by lactation but iPTH appears to be raised in lactating women (Specker, Tsang & Ho, 1991; Ardawi, Nasrat & A'Aqueel, 1997). For these reasons it was necessary to wait until the participating women had finished lactating before carrying out the last calcium load experiment. Inevitably this produced great variation in the timing of the post-pregnancy studies. However, almost all were performed within one year and there was no significant difference between the timing of the normal and IDDM post-pregnancy studies so comparisons are still likely to be valid. The prolonged wait also reduced compliance with the post-pregnancy study, and one woman in each group moved away from the area before stopping lactation. Post-pregnancy data are also compounded by the women being at different phases of their menstrual cycle or using oral contraception. It was impossible to prevent the use of oral contraception and it also proved difficult to try to bring up all the women during the same phase of the menstrual cycle because of the constraints of childcare and the women's work. For example they might only be able to come on a specific day when their partner or another person was available to provide babysitting. It was decided that it was more important to get the women to reattend at any time rather than lose their participation as a non-pregnant control.

Earlier work had shown that there was no significant difference in urinary sodium, potassium or calcium excretion between the two phases of the menstrual cycle and also that the concentration of iPTH was unchanged (Bisson, 1992). Plasma concentrations of renin, Aogen, Ang II and aldosterone are all known to fluctuate during the menstrual cycle with peak activity after ovulation (Brown *et al,* 1964 and Kaulhausen *et al,* 1978). More recent work looking at women on modern, low dose oestrogen (30mcg), combined oral contraceptives (COC) and women in the third trimester of pregnancy compared to controls has shown that both pregnancy and the COC produce a similar 3-4 fold rise in PRS (Derkx *et al,* 1986). Despite having a few women in each non-pregnant group on the COC this study was still able to shown a similar magnitude of rise in PRS between the non-pregnant women and the pregnant women. The same study by Derkx showed lower plasma concentrations of renin measured by an immunoreactive method in the women using COC compared with control women. In this thesis PRC was measured by reacting the renin in the sample with excess (heterologous) Aogen under standard pH and temperature conditions.

Therefore the differences in PRS between COC users and non-users was not relevant and the same applies to the increased PRS in pregnancy. In addition the main purpose of this study was to compare normal women with IDDM women and as both were rather disparate groups outside of pregnancy the effects of COC use and progesterone-only pill use may have cancelled each other out leaving the main difference of diabetic status.

Fasting before the study

Glucose ingestion is known to increase both UCE and UMgE (Lindeman, Adler, Yiengst & Beard, 1967; Lemann *et al,* 1970). In the same paper Lindeman *et al* showed that glucose ingestion decreased potassium excretion and that dietary protein led to similar increases in UCE and UmgE, whereas eating fat had no effect. For all these reasons it was important that the patients were fasted before the study. This could potentially have produced problems in the IDDM group; as it was only two women were hypoglycaemic prior to the start of one of the studies with plasma glucose values of 2.0mmol/L and 1.9mmol/L. One woman had to eat a slice of toast 90 minutes before the start of the 21 week experiment. The experiment was continued in this case because it was impossible for the woman to rearrange another morning for the study within the appropriate time frame. This particular woman did have higher than average IDDM values of FEK, FECa and FEMg at 21 weeks but her values were not the highest measured and did not significantly affect the study results overall.

4.2 Dietary study results

The gold standard for assessing dietary intake of a specified nutrient is to weigh all food and drink consumed over a given time period and then calculate amounts using a reference volume for the composition of foods. Studies such as these which are usually carried out on in-patients are highly disruptive to normal life and also extremely expensive. For these reasons it was decided to use a food frequency questionnaire to evaluate calcium intake. The questionnaire selected had previously been validated in 25 subjects, who not only completed the questionnaire with a dietician, but also kept a food diary over 8 days in which they recorded the weights of food and drink consumed, having previously been instructed in how to do this. The mean daily intake estimated from the questionnaire was 1134mg calcium and that derived from the diaries was 1173mg (Ryan *et al,* 1994). During this study the questionnaire was administered by the researcher, after training by a dietician. To increases accuracy food portion models were used.

Food frequency questionnaires have also been used to assess nutrient intake in pregnant women with similar backgrounds to the volunteers in this study (Robinson, Godfrey, Osmond, Cox & Barker, 1996; Rogers & Emmett, 1998). The former showed closest agreement between the questionnaire and food diaries for calcium intake while the latter found that pregnant women in the South West of England tended to consume slightly more calcium daily than the national daily recommendation for non-pregnant women.

The majority of studies on calcium supplementation in pregnancy have attempted to make some assessment of baseline calcium intake although this was not done in the largest of the South American studies (Belizan *et al,* 1991), nor in that of Lopez-Jaramillo *et al* (1989) who studied women in the Andes. Others used either food frequency questionnaires (Crowther *et al,* 1999) or 24 hour dietary recall methods (Belizan *et al,* 1983[a]; Villar *et al,* 1987; Knight & Keith, 1992; Levine *et al,* 1997). Average daily calcium intakes during pregnancy in this study were 1316mg in the normal women and 1574mg in the IDDM women with wide standard deviations in both. These values are very close to the American data presented by Levine *et al* (1997) whose calcium and placebo groups had intakes of 1113mg and 1135mg

respectively before supplementation, and again standard deviations were in the region of ± 700mg. The Australian study, which again looked at women from a developed country, had similar results of 1144mg in the calcium group and 1268mg in the placebo group, although, surprisingly, just over a quarter of all the women in this study reported intakes of less than 800mg per day (Crowther *et al* 1999). Unfortunately the Australian study did not report on the racial mix of their recruited women which could have accounted for this dietary discrepancy. This figure is closer to the South American dietary results quoted by Belizan *et al*, 1983[a] where average daily calcium intake was only 700mg.

Calcium intake in pregnancy in this thesis was in line with the Committee on Medical Aspects of Food Policy's, 1991 recommendations of at least 1250mg daily. Outside pregnancy, women in this study also took in more than the minimum recommendation. Therefore the women in this study are very different to the South American women, studied by Belizan's group, who have calcium deficient diets as defined by the British standard (Belizan *et al,* 1983[a], 1983[b], 1988 & 1991).

4.3 Blood pressure results

Measurement of blood pressure

It has been known for many years that BP has a diurnal variation (Brown, 1930). BP in pregnancy is also affected by the position of the patient; if it is taken with the mother lying supine 70% have at least a 10% fall compared to seated values, and in 8% it falls by 30-50% (Holmes, 1960). To minimize these differences our readings were all taken between 09.00hr and 12.00hr and with the patients sitting. BP recording in pregnancy is made even more problematic by the fact that Korotkoff sounds may persist to zero because of peripheral vasodilation. For this reason it was once recommended that Korotkoff IV should be used to measure the DBP in pregnancy (Petrie, O'Brien, Littler & de Sweit, 1986; World Health Organization, 1987; Davey & MacGillivray, 1988)) and this was used in this study. However while this study was on-going this advice changed and it is now recommended that Korotkoff V be generally used, with Korotkoff IV being recorded in the very rare cases when Korotkoff V does in fact persist to zero (de Swiet & Shennan, 1996). To minimize inter-observer error most of the BP measurements were taken by the same observer with a minority being carried out by one midwife who had been instructed in the technique to use. As neither of these observers was blind to the diabetic status of the woman this could have biased the results. This would have been unacceptable if the primary purpose of this thesis had been to look at BP measurements in IDDM and normal women. However, as this was only done as a minor part of the study, and there was no funding to provide an unbiased person to take BP readings it was the best that could be achieved. Alternatives would have been to use either an automated sygmomanometer or the Hawksley random zero sphygmomanometer to reduce observer error or bias. Unfortunately neither of these machines is problem free. Automated machines tend to underestimate the DBP, especially during pregnancy, because of the discrepancy between Korotkoff IV and V mentioned above. The random zero monitor has also been shown to be more inaccurate than standard sphygmomanometers (O'Brien, Mee, Atkins & O'Malley, 1990).

Results

Whilst this study did not have the statistical power to comment on the incidence of PE in IDDM pregnancy it was interesting that none of the normal women developed PE

but three of the IDDM women did. This gives an incidence of 15% for PE in IDDM pregnancy, similar to 14.4% found by Brudenell (1982) and 9.9% reported by Garner *et al* (1990), and higher than the incidence reported in non-diabetic women which varies from 1% to 5%, or more depending on the definition used.

This study did not show any effect of a daily 1.5g calcium supplement on BP. This contradicted several reported studies (Belizan *et al,* 1983[a]; Villar *et al,* 1987; Lopez-Jaramillo *et al,* 1989; Belizan *et al,* 1991; Knight & Keith, 1992). This study was very small with only 10 normal and 10 IDDM women assigned to each arm of the trial. However, two of the studies listed above were also extremely small with Belizan's work of 1983 showing an effect of both 1g and 2g calcium supplements on 11 women each and Knight & Keith's work looking at 20 women with PIH half of whom received 1g calcium. This thesis looked at women of mixed parity with 17 out of 40 being multiparous and therefore at reduced risk of PE. In this respect this study was compatible with Belizan's initial report of 1983 which also studied women of mixed parity. The remaining studies mentioned above only considered primigravidae.

The women in this study were all receiving a good diet with an adequate intake of calcium for pregnancy as suggested by the United Kingdom Committee on Medical Aspects of Food Policy (1991); that is at least 1250mg/day. In contrast the papers cited above had looked at women with below average calcium intakes; the 1983 paper from Belizan's group looked at urban Guatemalan women on a average diet containing 700mg calcium per day. Knight & Keith (1992) looked at women from Mississippi but the hypertensive white women in this trial were only taking 810mg calcium daily and the black women with PIH even less – 351mg per day on average. This dietary difference is the most likely reason why the women in this study did not benefit from calcium supplements.

Four years after work on this thesis began the results of the National Institutes of Health of the USA trial of calcium supplementation to prevent PE were published and showed no beneficial effect of 2g/day calcium supplements for normal, healthy, nulliparous women whose diet already contained adequate calcium (Levine *et al,* 1997 (table 4.1).

Table 4.1 Incidence of hypertensive disorders during pregnancy according to treatment group (after Levine *et al*, 1997)

Condition	Calcium N = 2295	Placebo N = 2294	Relative risk (95% CI)
Pre-eclampsia:	158 (6.9)	168 (7.3)	0.94 (0.76-1.16)
- mild	108 (4.7)	109 (4.8)	0.99 (0.76-1.28)
- severe	50 (2.2)	59 (2.6)	0.85 (0.58-1.23)
Pregnancy-associated hypertension:	351 (15.3)	397 (17.3)	0.88 (0.78-1.01)
- mild	335 (14.6)	381 (16.6)	0.88 (0.77-1.01)
- severe	16 (0.7)	16 (0.7)	1.00 (0.50-1.99)
All hypertensive disorders	509 (22.2)	565 (24.6)	0.90 (0.81-1.00)

Results are given as numbers (%) of women and relative risks with 95% confidence intervals. The relative risks are those for a woman in the calcium group compared to a woman in the placebo group

In 1999, Crowther *et al* published their results which showed a beneficial effect of 1.8g daily calcium supplementation in preventing PE in 456 nulliparous, Australian women (relative risk 0.44[95%CI 0.21-0.90]). This appears to contradict the much larger American trial but the Australian trial was halted because of a lack of funding when they had recruited less than half of the women suggested by their original power calculations and therefore their results need to be interpreted with caution. A surprisingly large number of women in the Australian trial had low calcium intakes with 29.3% in the calcium group and 27.7% in the placebo group consuming less than 800g calcium per day. Unfortunately no comment was made on the ethnicity of the subjects in the Australian trial which may have contained large numbers of women of aboriginal origin who may well have a less good diet than Australians of European or Asian descent.

The results for normal women presented in this thesis are in keeping with the bigger prospective, randomized, double-blind, placebo-controlled trial from America which

has shown that the prevention of PE is not easily obtained with this simple dietary manipulation in women who are already calcium-replete (Levine *et al,* 1997).

When normal and IDDM women were compared in this study the normal women had a fall in DBP during the second and early third trimester with a gradual rise towards the end of pregnancy as first described by MacGillivray, Rose & Rowe (1969). This "mid-trimester" fall was not apparent in the IDDM women. Similar findings were reported by the 'Diabetes in early pregnancy study' which enrolled 312 IDDM women and 356 control women within 3 weeks of conception and found no difference in either SBP or DBP between the groups at 6 weeks' gestation. Although not all the women in this study had their BP measured at each time point almost 250 IDDM women and 300 normal women did have their BP measured during the second trimester; the normal women showed the expected fall but the IDDM women did not (Peterson, Jovanovic-Peterson, Mills, Conley, Knopp, Reed, Aarons, Holmes, Brown & Van Allen, 1992).

This fall in DBP in the middle trimester of normal pregnancy is likely to be the result of peripheral vasodilation which reduces the peripheral vascular resistance more than can be compensated for by the pregnancy-induced rise in cardiac output. Thus the failure of the IDDM women to show such a fall in DBP could either be as the result of an increased cardiac output or because of a reduced fall in peripheral resistance.

Cardiac output is a function of left ventricular stroke volume and the heart rate. In 1986 Airaksinen, Ikaheimo, Salmela, Kirkinen, Linnaluoto & Takkunen performed echocardiography on 17 pregnant women with IDDM and 11 normal pregnant women who acted as controls. The mean duration of diabetes was 14 years, exactly the same as in this thesis. The women were studied prospectively in each trimester and post-partum. The authors found that the IDDM women had slightly smaller left ventricles than the normal women when not pregnant and that they also had less of a pregnancy-induced increase in left ventricular size and stroke volume. As they also had a smaller increase in heart rate during pregnancy the overall pregnancy associated rise in cardiac output was only 1.3L/min in the IDDM women compared to 3.4L/min in the normals. Therefore the failure of DBP to fall mid-trimester in the IDDM women appears not to be caused by an increased cardiac output.

Peripheral resistance is controlled by both the autonomic nervous system and by various substances which act directly on the blood vessels. Ang II is the most potent vasoconstrictor known and the role of iCa^{2+} in vascular contraction and relaxation has been discussed already in the introductory section of this thesis. Other substances which produce local vasodilation are the end-products of tissue metabolism such as hydrogen ions, potassium, lactate, carbon dioxide and prostacyclin. As discussed in greater detail later (Section 4.6, p197) this thesis found decreased PRC in IDDM pregnant women. If all other considerations were equal this would tend to produce lower levels of Ang II in the IDDM women and therefore vasodilation and a fall in BP. This thesis also did not show any change in serum iCa^{2+} concentrations in IDDM women.

Prostaglandins were not measured in this thesis. As mentioned above it is likely that the fall in DBP which occurs in normal pregnancy in caused principally by 'flooding' of the circulation by vasodilators – chief among these being prostacyclin. The urinary excretion of prostacyclin, which equates directly to the its serum concentration but is technically easier to measure, rises four-fold by 20 weeks in normal pregnancy and remains at this level until delivery. In contrast it only rises two-fold in pregnancies which subsequently become complicated by PIH (Fitzgerald, Entman, Mulloy & Fitzgerald, 1987). There is now increasing evidence that prostacyclin production is impaired in diabetic pregnancy (Kuhn, Botti, Cherouny & Demers, 1990). This will result in reduced peripheral vasodilation and hence less of a fall in BP.

The blood vessels of pregnant IDDM (n=10) women have also been shown to be 'stiffer' than those of normal pregnant women (n=20) in a study which used ultrasound to determine a stiffness index and hence the elasticity of the maternal aorta and carotid arteries (Hu, Bjorklund, Nyman & Gennser, 1998). This difference could also limit peripheral vasodilation and lead to a failure of BP to fall as much in IDDM pregnancy as in normal pregnancy.

The failure of the pregnant IDDM women to show the usual mid-trimester fall in DBP may also be because of impaired autonomic nervous system activity. Recent use of 24 hour ambulatory blood pressure monitoring (ABPM) has shown that non-pregnant IDDM subjects with normal BP by conventional recording and no evidence of renal

impairment, defined by no microalbuminuria, are less likely than control subjects to have an overnight fall in BP (Spallone, Gambardella, Maiello, Barini, Frontoni & Menzinger, 1994; Gilbert, Phillips, Clarke & Jerums, 1994; Khan & Couper, 1995). In one of these studies a score for autonomic dysfunction was determined (Spallone *et al,* 1994) and there was a positive correlation between this score and the degree of abnormality in the 24hr ABPM profile. Autonomic nervous function was not studied in this thesis. In a study reported in 1987 autonomic function was measured during and after pregnancy in 25 IDDM women and 10 control women. No differences were found between the groups outside of pregnancy but autonomic function was impaired in the IDDM group during pregnancy (Airaksinen, Salmela, Ikaheimo, Kirkinen, Linnaluoto & Takkunen, 1987). Loss of diurnal variation in BP in pregnant women with PE has recently been reported (Halligan, Shennan, Taylor & de Swiet, 1997) and raises interesting possibilities about the increased incidence of PE in IDDM pregnancies.

It was disappointing that although, on initial inspection of the data, calcium supplementation appeared to lower both SBP and DBP in the IDDM women this affect disappeared when the data were reanalysed as percentage changes from the BP at 20 weeks when the women were randomized to calcium or placebo. Although the IDDM women in this study were all taking diets adequate in calcium as defined above it had been hypothesized, prior to starting the study, that intestinal absorption of calcium might be impaired in IDDM making these women effectively calcium-deficient and therefore more likely to have shown an effect of calcium supplementation.

Calcium absorption, as discussed in the introductory section of this thesis (Section 1.2A, p12) is regulated by 1,25-DHCC, production of which is regulated by iPTH. Levels of 1,25-DHCC rise in normal pregnancy (Kumar, Cohen, Silva & Epstein, 1979). To our knowledge vitamin D levels have not been studied in IDDM pregnancy. In 1972 Schneider & Schedl reported decreased absorption of calcium in the duodenum of diabetic rats compared to normal rats; they did not directly measure either iPTH or 1,25-DHCC. Subsequent work found plasma levels of 1,25-DHCC to be low in diabetic rats (Hough, Russell, Teitelbaum & Avioli, 1982). Human studies have shown conflicting results: in 1982, Christiansen, Christensen, McNair, Nielsen

& Madsbad found normal levels of 1,25-DHCC in adult male IDDM subjects, although levels of 25-HCC and 24,25-DHCC were reduced. A few years later the finding of normal levels of 1,25-DHCC in Caucasian IDDM subjects was confirmed although the same workers found reduced levels of 1,25-DHCC in poorly-controlled Bantu IDDM subjects (Nyomba, Bouillon, Bidingija, Kandjingu & de Moor, 1986). The IDDM women in our trial were well controlled, especially during the pregnancy arm of the study and therefore likely to have normal levels of 1,25-DHCC. It was extremely unfortunate that promised funding was withdrawn so that 1,25-DHCC could not be measured in this study. It was interesting that in the tiny group of women in whom it was measured at 31 weeks gestation there was no difference between the normal and IDDM women but obviously this finding would need to be validated in larger numbers.

1,25-DHCC is also produced from 25-HCC in the placenta (Weisman, Harell, Edelstein, David, Spirer & Golander, 1979; Whitsett, Ho, Tsang, Norman & Adams, 1981). 1,25-DHCC levels have been found to be low in PE (August, Marcaccio, Gertner, Druzin, Resnick & Laragh, 1992 and Graves, Wood, Brown & Seely, 1994) and it has been suggested that this is due to impaired placental production of the vitamin, yet another sign of the inadequate placentation which occurs in pregnancies which are complicated by PE. 1,25-DHCC was not measured in any of the three women who developed PE in this study. However, their iCa^{2+} levels were not low, suggesting that they were not calcium deficient.

4.4 Biochemistry results

In the non-pregnant women fasting serum Mg, PO_4, urate and albumin concentrations were all significantly less in the IDDM women; at 21 weeks gestation these variables were identical in the two groups. This implies that the IDDM women do not respond to pregnancy in a similar way to the normal women. Concentrations of albumin are known to fall during normal pregnancy because of changes in plasma volume which rises progressively during pregnancy, plateauing during the last 8 weeks or so (Pirani, Campbell & MacGillivray, 1973). Robust experimental data on the plasma volume in IDDM pregnancy is not available but the consensus of opinion is that it probably does not increase to the same extent. The concentrations of other plasma electrolytes are also known to fall in normal pregnancy, partly because of dilution due to the increased plasma volume and partly as a result of increased renal excretion. The GFR increases soon after conception, reaching 60% above non-pregnant levels by 16 weeks and remaining there for the rest of the pregnancy (Davison, 1974). Tubular function is also affected with some substances being less efficiently reabsorbed during pregnancy. In 1977, Dunlop & Davison reported the excretion of urate was increased in normal pregnancy leading to a fall in plasma levels.

This thesis found an increase in creatinine clearance and falls in serum sodium, potassium, creatinine, Mg, PO_4, urate and albumin in normal pregnancy, compared to the non-pregnant state, which is in keeping with the finding of others mentioned above. In contrast in the IDDM women there was no fall in serum sodium, Mg, PO_4 or urate and although creatinine clearance increased from the non-pregnant value this was not significant. One explanation for this is that the IDDM women have some degree of impaired renal function compared to the normal women and that this difference is exacerbated when their kidneys are presented with the increased renal plasma flow which occurs in pregnancy as described by Davison & Dunlop, 1980. However, only one of the IDDM women studied had a second trimester urinary albumin:creatinine ratio outside the normal range and when creatinine clearances were compared outside pregnancy there was no difference between the normal and IDDM women. Also the results of this study showed the fractional excretion rate of sodium was not affected by diabetic status outside pregnancy and during pregnancy it was raised in the IDDM women compared to normal. Therefore the failure of the

concentration of serum sodium to fall in IDDM pregnancy must be caused by a smaller increase in plasma volume rather than impaired renal excretion of sodium.

There were also no differences in the 24hr excretion of PO_4 between the normal and IDDM women at any gestation and in fact during pregnancy the $FEPO_4$ was increased in the IDDM women compared to the normal women implying yet again that serum PO_4 concentration did not fall in IDDM pregnancy because of less increase in plasma volume. Phosphate excretion will be discussed in greater detail below in the subsection relating to iPTH results.

When serum Mg is considered the situation is more complicated. In normal pregnancy serum Mg has been shown to fall progressively between 6 and 38 weeks ($p<0.001$) and as this fall parallels the fall in serum proteins it is likely to be due to haemodilution (Seydoux, Girardin, Paunier & Beguin, 1992). This thesis showed a similar fall. Outside pregnancy McNair, Christiansen, Madsbad, Lauritzen, Faber, Binder & Transbol (1978[b]) reported very significantly lower ($p<0.001$) levels of serum Mg in IDDM subjects compared to normal controls. This finding was subsequently confirmed by Mather, Nisbet, Burton, Poston, Bland, Bailey & Pilkington (1979) and they showed a significant ($p<0.001$) negative correlation between serum Mg and plasma glucose. Other workers went on to confirm low serum Mg in diabetic subjects and to show that the worse the degree of diabetic control the lower the serum Mg (Sjogren, Floren & Nilsson, 1986).

It appears that serum Mg is low in IDDM subjects because of increased renal loss of Mg (McNair, Christensen, Christiansen, Madsbad & Transbol, 1982; Olukoga, Adewoye & Erasumus, 1989) and in anaesthetized rats treatment with insulin has been shown to reduce the renal loss of Mg (Anwana & Garland, 1990). Recently a group of Danish workers showed that a 3 month period of improved glycaemic control in 49 subjects, achieved by frequent outpatient review and adjustment of insulin dosage, similar to the intensity of treatment given to the pregnant IDDM women in this study, reduced the urinary loss of Mg but did not change the average serum Mg concentration (Djurhuus, Henriksen, Klitgaard, Blaabjerg, Thye-Ronn, Altura, Altura & Beck-Nielsen, 1999).

The results presented in this study of lower concentrations of serum Mg and raised 24 hour urinary Mg excretion in the non-pregnant IDDM volunteers compared to non-pregnant controls are in keeping with the findings described above. Inevitably there is much less published data on Mg levels in IDDM pregnancy. In 1985 Wibell, Gebre-Medhin & Lindemark reported lower serum Mg in IDDM pregnant women compared with normal pregnant controls; Kuoppala (1988) found similar levels of serum Mg in pregnant controls and IDDM women and also in a small (n=14) number of women with gestational diabetes. A different study found no difference between extracellular total Mg in normal and diet-controlled gestational diabetic pregnancies but reduced intracellular free Mg in the gestational diabetic group (Bardicef, Bardicef, Sorokin, Altura, Altura, Cotton & Resnick, 1995).

In view of this small number of studies on serum Mg concentrations in IDDM pregnancy and the discrepancies in their results, this thesis, looking both *prospectively* and *longitudinally* at serum Mg, in 20 IDDM women, is valuable. This thesis has found that serum Mg was the same in both the second and third trimesters in normal and IDDM pregnancy and although the 24 hour excretion of Mg was higher in the IDDM women at both 21 and 31 weeks this difference was not significant. As mentioned above others groups have found a significant negative correlation between plasma glucose and serum Mg and this study also showed this association both during and outside of pregnancy. During pregnancy the IDDM women in this study had better glycaemic control and this is likely to explain the lack of difference between them and the normal women when serum Mg and urinary Mg excretion are compared.

Outside pregnancy approximately 30% of filtered Mg is reabsorbed in the PCT, 50-60% in the Loop of Henle and the remainder is excreted. The mechanism of Mg reabsorption is not understood but iPTH is not though to be involved (Hosking, 1998). This study showed no correlation between the FEMg and iPTH concentration in the normal non-pregnant women but significant negative correlations between these variables at both 21 weeks ($p<0.01$) and 31 weeks ($p<0.01$) were observed. Similar negative correlations were observed between these variables in the IDDM women when not pregnant and at 21 weeks gestation. Although these results initially appear to contradict the established view that iPTH is not involved in the physiological control of Mg excretion it is more likely that the correlations reported in this thesis are

a spurious association produced by the confounding effect of the standard meal and oral calcium load. This study clearly showed a very significant effect ($p<0.001$) of the standard meal and oral calcium on the FEMg which increased by approximately 3-fold over the 4 hours from the meal in both groups of women at all three gestations (table 3.39 and fig. 3.41). Over the same time the oral calcium load markedly suppressed levels of iPTH (see next section). Therefore, in this particular study, it is not surprising to find negative correlations between concentrations of iPTH and the FEMg.

4.5 iPTH results and the effect of the oral calcium load on serum concentrations of iPTH, iCa^{2+} and PO_4 and the FE rates of these ions

As discussed in detail in the introduction to this thesis (section 1.2C, p14) the concentration of iPTH is known to be controlled predominantly by the concentration of serum iCa^{2+}. iPTH acts to increase levels of serum iCa^{2+} by two main mechanisms. Firstly, via 1,25-DHCC, the proportion of dietary calcium which is absorbed in the gut is increased and secondly iPTH acts directly on the PCT of the kidney to increase calcium reabsorption while PO_4 excretion is increased. This study did not look at gut absorption of calcium but it did study the relationship between serum iCa^{2+} and the concentration of iPTH and also the effect of altering both these variables on the excretion of calcium and PO_4. Pregnancy-induced changes in these factors and differences between normal and IDDM subjects during and outside pregnancy are discussed in this section. Relationships between iPTH and the RAS are discussed in the following section.

Several studies have found reduced levels of serum iCa^{2+} in subjects with IDDM (McNair, Fogh-Anderson, Madsbad & Christensen, 1983; Schwarz, Sorensen, Momsen, Friis, Transbol & McNair, 1992). However, the data are not all in agreement with Witt, White, Santiago, Seino & Avioli, (1983) reporting normal levels of serum iCa^{2+} in IDDM although the individuals in this study were not directly compared with controls. Fasting non-pregnant serum iCa^{2+} levels in this study were the same in both IDDM and normal women.

There is no consensus as to what happens to serum iCa^{2+} during pregnancy. Some report it as falling slightly (Tan *et al,* 1972; Pitkin & Gebhardt, 1977; Pitkin *et al,* 1979), others have found that it rises (Reitz *et al,* 1977) and further studies have found no change (Gertner *et al,* 1986; Roelofsen *et al,* 1988; Dahlman, Sjoberg, Bucht, 1994; Seely *et al* 1997). Another group reported a more complex picture with an overall rise in pregnancy compared with 12 months post-partum but a slight fall in the latter part of the third trimester (Rasmussen *et al,* 1990). Only four of these studies (Pitkin *et al,* 1979; Rasmussen *et al,* 1990; Dahlman *et al,* 1994; Seely *et al,*1997) used the same women throughout the study so that each woman gave repeated blood samples and acted as her own non-pregnant control; the others used groups of

different women at different gestations and this may partly account for the discrepancy in their results. Although the longitudinal studies are likely to give a more accurate picture of pregnancy-induced changes in serum iCa^{2+} the post-partum data in the studies of both Pitkin *et al* (1979) and Seely *et al* (1997), are confused by the fact that some of the women were still lactating (20 of 30 women and 8 of 23 women respectively) when the blood samples were drawn. Pitkin compared ionized calcium levels in the lactating and non-lactating women and found no difference between the groups but this work has not been repeated with larger numbers (Pitkin *et al* 1979). The studies by Rasmussen *et al* (1990) and Dahlman *et al,* (1994) did not mention whether or not the women were lactating when they gave blood samples.

The study described in this thesis was a longitudinal, prospective trial in which each woman acted as her own non-pregnant control, only after she had discontinued breast-feeding, and thus the criticisms mentioned above do not apply although there were considerable time delays between the pregnant and not pregnant samples (median 40 weeks in the normal group and 29 weeks in the IDDM group). However it is unlikely that levels of serum iCa^{2+} change significantly over this sort of time period. The results presented earlier confirm that serum iCa^{2+} does not change in pregnancy. The little published work on serum iCa^{2+} in IDDM pregnancy indicates that concentrations are not altered by pregnancy (Cruikshank, Pitkin, Reynolds, Williams & Hargis, 1980; Cruikshank *et al,* 1983). Results in this thesis agree with this. Since a stable extracellular concentration of iCa^{2+} is important for many physiological functions, as described in the introductory section, it makes sense that this stability is maintained in both normal and IDDM pregnancy as demonstrated in this thesis. However it seems that the homeostatic mechanisms to maintain stable serum iCa^{2+} are different in pregnancy.

Pregnancy was once though to be a "state of physiological hyperparathyroidism" but this was before assays which measured iPTH were available. Now different groups have reported either no change in pregnancy (Mimouni, Tsang, Hertzberg, Neumann & Ellis, 1989; Saggese, Baroncelli, Bertelloni & Cipolloni, 1991) or lower values during pregnancy, compared to postpartum (Rasmussen *et al,* 1990; Ardawi *et al,* 1997) with none finding increased iPTH. This study showed that iPTH tended to be lower in normal pregnancy than in the same women outside pregnancy but this

difference failed to reach statistical significance. It found no difference between values at 21 and 31 weeks gestation and showed no effect of chronic calcium supplementation on iPTH concentration. If, as all these studies and this thesis, suggest iPTH is not increased during pregnancy then there must be another mechanism by which the mother is able to obtain the 700mmol of calcium required to create a healthy term baby especially as the UCE increases markedly during pregnancy as mentioned in section 1.2F, p18. The most likely explanation is that the proportion of dietary calcium absorbed rises during pregnancy because of the increased levels of 1,25-DHCC which have been shown to occur in pregnancy (Kumar *et al,* 1979; Whitehead *et al,* 1981; Reddy *et al,* 1983; Gertner *et al* 1986; Verhaeghe & Bouillon, 1992; Ardawi *et al,* 1997; Seely *et al* 1997).

Much of the additional vitamin D found in pregnancy is produced in the placenta. The ability of human decidual tissue to generate 1,25-DHCC and of human placenta to generate both 1,25-DHCC and 24,25-DHCC *in vitro* was first reported in 1979 (Weisman *et al*). Subsequently Whitsett *et al* (1981) showed that human placenta could also synthesize 1,25-DHCC *in vitro.* As well as directly producing vitamin D the placenta secretes a protein hormone, PL, which increases *in vitro* renal 1-alpha-hydroxylase activity (Spanos *et al,* 1981). Additionally PL stimulates the production of insulin-like growth factor 1 (IGF-1) in the mother and the fetus and this also increases renal 1,25-DHCC production (Caverzasio *et al* 1990). Oestrogen, as mentioned above (section 1.2B, p13) also stimulates renal 1-alpha-hydroxylase activity and is a further cause of the raised levels of vitamin D found in pregnancy.

Levels of 1,25-DHCC are known to be reduced in pregnancies complicated by both PIH (Frolich, Rudnicki, Storm, Rasmussen & Hegedus, 1992) and PE (August, Maracaccio, Gertner, Druzin, Resnick & Laragh, 1992). It has been hypothsized that this is caused by reduced 1,25-DHCC production by the abnormal placentae found in hypertensive pregnancies. Placentation in IDDM pregnancy is likely to be somewhat different to normal placentation and it would have been interesting if this thesis had been able to show low levels of 1,25-DHCC in IDDM pregnancy thus demonstrating a similar impairment in calcium metabolism in IDDM and PE pregnancies. Although the results presented earlier show an 11% reduction in levels of 1,25-DHCC in IDDM pregnancy in the third trimester, as only 7 women were analysed in each group

because of financial problems, this result was not significant and so conclusions cannot be drawn from it. Very little is known about vitamin D levels in human IDDM pregnancy; in 1988 Kuoppala published a cross-sectional study which found lower levels of both 25-HCC and 1,25-DHCC in IDDM pregnancy compared to normal pregnancy. Later prospective studies found reduced levels of 1,25-DHCC (Mimouni *et al,* 1989) and 25-HCC in IDDM pregnancy (Martinez, Catalan, Balaguer, Lisbona, Quero, Reque & Pallardo, 1991). The findings of this thesis appear to agree with these studies.

The findings of lower levels of iPTH in IDDM pregnancy reported in this thesis were very interesting. As with 1,25-DHCC little work has been done on levels of PTH in IDDM pregnancy. Using a C-terminal assay for PTH Cruikshank, Pitkin, Varner, Williams & Hargis (1983) reported reduced concentrations of PTH in IDDM pregnancy and a later study, which like this thesis, measured iPTH (Mimouni *et al,* 1989) found low-normal concentrations of iPTH in IDDM pregnancy compared to normal pregnancy. Others have suggested that the reduced values of iPTH found in IDDM pregnancy could be caused by reduced concentrations of serum Mg (Mimouni *et al*, 1989). Severe Mg deficiency is known to impair iPTH secretion (Anast *et al*, 1976) but more physiological reductions in serum Mg do not appear to have this effect (Toffaletti, Cooper & Lobaugh, 1991). This thesis did not find either reduced concentrations of serum Mg in IDDM pregnancy or a positive correlation between serum Mg and iPTH levels in either the normal or IDDM women. There were significant negative correlations between serum Mg and iPTH at 21 weeks and outside pregnancy in the IDDM women (fig. 3.49, p151); when serum iCa^{2+} was controlled for using partial correlation analysis this apparent negative correlation disappeared in the non-pregnant IDDM women. However at 21 weeks the negative correlation remained highly significant after controlling for serum iCa^{2+}. No correlation between serum Mg and serum iPTH was demonstrable at 31 weeks in the IDDM patients regardless of whether or not serum iCa^{2+} concentrations were considered. In renal dialysis patients chronic hypermagnesaemia has been shown to decrease PTH secretion (Navarro, Mora, Jimenez, Torres, Macia & Garcia, 1999). IDDM patients, as discussed above, tend to be in a state of chronic Mg deficiency which was reversed in the patients in this study during pregnancy when their glycaemic control improved. Therefore for IDDM women pregnancy is a state of

relative hypermagnesaemia and this could cause the significantly reduced iPTH levels found at 21 weeks. By 31 weeks the correlation between these variables had disappeared possibly because the parathyroid glands had reset to higher levels of serum Mg by receptor down-regulation.

Oral calcium loading

As expected the oral calcium load produced a significant increase in serum iCa^{2+}. This increase was similar in the normal and IDDM women and was of the same order of magnitude at all three gestations studied. The UCE also increased as expected in both groups of women at all three gestations. Both variables plateaued at four hours after the oral calcium. Work done by others has shown similar increases in both of these variables which persist up to four hours after oral calcium (Broadus, *et al,* 1978; Kent *et al,* 1991). Broadus (1978) extended his experiments to six hours and showed a later fall in UCE.

It was mentioned above that PO_4 excretion is increased by iPTH. When the overall amount of PO_4 excreted per 24 hours was compared this was the same in the normal and IDDM women and was not affected by pregnancy. However the fasting fractional excretion of PO_4 was markedly raised in the IDDM women at both 21 ($p<0.05$) and 31 ($p<0.01$) weeks gestation (fig. 3.18). This difference between the two groups of women was maintained during the calcium load experiments with IDDM $FEPO_4$ being higher than normal $FEPO_4$ at all times. As iPTH was generally lower in the IDDM women in this study an additional factor must be involved to explain their raised $FEPO_4$. Ingestion of both glucose and galactose is known to increase urinary PO_4 excretion within an hour (Lindeman *et al,* 1967) possibly because raised plasma glucose levels produce an osmotic diuresis and more PO_4 is filtered in the kidney. The standard breakfast in this study contained both glucose and galactose and therefore inevitably this had a confounding effect on the $FEPO_4$. This study showed strong positive correlations between plasma glucose and the $FEPO_4$ in the IDDM women at all times and this is likely to be the cause of their raised $FEPO_4$.

Following the oral calcium load in the non-pregnant women the $FEPO_4$ fell steadily (fig. 3.39) in both the normal and IDDM women with the IDDM women having higher values at all times as described above. During pregnancy the $FEPO_4$ also fell

after the oral calcium load although values for hour 1 were actually higher than the basal hour; this pattern was identical in both groups of women and at both study gestations (fig. 3.39). This fall in $FEPO_4$ corresponds with suppression of iPTH and a rise in FECa and was to be expected after the oral calcium.

As mentioned above ingestion of both glucose and galactose is known to increase urinary PO_4 excretion within an hour (Lindeman *et al,* 1967). During pregnancy the plasma glucose rises more, at one hour, in response to a glucose load than in non-pregnant individuals (Hatem, Anthony, Hogston, Rowe & Dennis, 1988). The standard breakfast in this study contained both glucose and galactose and therefore inevitably this had a confounding effect on the $FEPO_4$ and may have accounted for the initial rise in $FEPO_4$ in the pregnant women. Beyond this time plasma glucose tended to fall back towards fasting levels and therefore other factors such as the FECa and the suppressed serum iPTH were quantitatively more important in controlling the $FEPO_4$ for the remainder of the calcium load experiments.

Ca supplementation

The calcium supplements which half of the women were receiving by 31 weeks did not affect serum iCa^{2+}or the UCE during either the fasting hour or in the four hours after the oral calcium load. This was not unexpected as the women did not take their supplements on the morning of the experiment. As this study and those of Broadus, *et al* (1978) and Kent *et al* (1991) have shown the changes induced by acute calcium loading are immediate but last only a few hours and so effects of the calcium supplements on either serum iCa^{2+} levels or UCE would not be expected to persist after an overnight fast.

Unexpectedly the subgroup of women receiving the calcium supplements did not show a significant increase in the 24 hour urinary excretion of calcium. This is likely to be because the 24 hour excretion of calcium was measured in mmol/24 hours whereas the increases in UCE produced by the oral calcium loading were at maximun only 8mcmol/min, equivalent to 1mmol/24 hours. Small differences like this can be easily lost in an inaccurate 24 hour urine collection. As was pointed out in the Introduction the majority of ingested calcium is excreted in the faeces; had this study measured faecal excretion of calcium one would have expected this to be raised in the

supplemented group. The failure of calcium supplements to alter the concentration of serum iCa^{2+} tends to support the hypothesis that calcium supplementation will not alter BP in women who are already receiving a diet adequate in calcium as it is difficult to suggest a mechanism for and effect on BP when serum iCa^{2+} is unchanged.

Serum iCa^{2+} in the IDDM women who developed PE was the same as in the other IDDM women. As none of the 3 future pre-eclamptic women were hypertensive at the time when the blood samples were taken it is impossible to comment about levels of serum iCa^{2+} in PE pregnancy.

4.6 Plasma renin concentration and plasma renin substrate results

The results presented in this thesis confirm numerous earlier studies showing that both PRC and PRS are increased in normal pregnancy compared with data from non-pregnant individuals (Skinner *et al*, 1972; Weir *et al*, 1975; Baker *et al*, 1990). However this study was more concerned with differences between normal and IDDM subjects. An overview of the RAS in IDDM has been given in the introductory section of this thesis (section 1.3H, p48). In summary levels of PRS appear to be unaltered in IDDM but overall PRC levels are low with a greater proportion of inactive prorenin to active renin being found in diabetic subjects.

PRC results

Fasting PRC in the IDDM women studied was not lower than in the normal women outside pregnancy and this contradicts established work on non-pregnant subjects (Day *et al*, 1975; Luetscher *et al*, 1985). This could be a type II error caused by small numbers and, as not all of the 40 volunteers could be persuaded to return after pregnancy, numbers in the non-pregnant group are smaller than in the pregnant groups. Renin levels have been shown to be normal in young IDDM subjects with short (3-5 yrs) duration of disease (Wilson & Leutscher, 1990) but this does not account for the findings in this study as the average duration of disease in those who returned after their pregnancies was 13 years. However the IDDM subjects recruited to this study were relatively young, well-controlled and had very few diabetic complications and it is likely that there will be less differences between a diabetic group like this and non-diabetic subjects. After the standard breakfast PRC was lower, although not significantly so, in the IDDM subjects, compared to the non-diabetic women, and this is in agreement with the findings of others.

During pregnancy this study did show reduced PRC in the IDDM women compared to the normal group. Very little work has previously been done on PRC in IDDM pregnancy. In 1982, Broughton Pipkin, Hunter, Oats & O,Brien reported raised levels of PRC in 7 normotensive diabetic patients of whom 6 had been insulin-dependent before the pregnancy and compared them to 9 normal pregnant women. The discrepancy between these results may be explained by smaller numbers in the latter study and differences in patient selection. The women studied by Broughton Pipkin *et*

al 1982 were all primigravidae whereas in this study 6 of 20 IDDM women were multigravid. As multigravid women tend to have a greater increase in plasma volume during pregnancy they would be expected to have greater suppression of their RAS. However more of the normal women studied in this thesis were multigravid (11 out of 20) and still PRC was higher in the normal than IDDM women.

Higher plasma glucose concentrations are an obvious difference between groups of normal and IDDM subjects. However, as discussed in the introductory section of this thesis, reasons for low renin in IDDM are likely to be multifactorial and it would be simplistic to expect a strong correlation between PRC and plasma glucose. Therefore it was not surprising to find no relationship between these two variables in either group of women at most of the study gestations. What was surprising was the apparently very significant ($p<0.01$) correlation between PRC and plasma glucose in the normal women in the third trimester. It has been shown that the plasma glucose response to a 75g oral glucose tolerance test is greater in women in the third trimester compared with either women in the second trimester or with women outside pregnancy (Hatem *et al,* 1988). Extrapolating from this women in the third trimester of pregnancy will have higher plasma glucose levels than "normal" after meals and this could produce an osmotic diuresis. If this situation remained unchecked plasma volume would fall with serious consequences for the mother and fetus. The rise in PRC in response to rising plasma glucose which we have demonstrated would act to conserve plasma volume.

Chronic calcium supplementation did not affect PRC. This is in agreement with work done on dogs (Kotchen *et al,* 1974). Theoretically calcium could stimulate renin production by inhibiting sodium reabsorption in the kidney tubules but, as there was no difference in either serum sodium or the fractional excretion of sodium between the supplemented and non-supplemented women in this study, it is not surprising that PRC was unaffected too.

In this study there was a negative correlation ($p=0.01$) between serum iCa^{2+} and PRC in the normal women outside pregnancy which confirms the findings of others that a fall in extracellular iCa^{2+} concentration leads to an increase in renin secretion. This is discussed in detail in the introductory section of this thesis and so is merely

summarized here. Early experiments showed that the infusion of both calcium chloride and calcium gluconate into anaethetized dogs reduced the rate of renin secretion (Kotchen *et al*, 1974; Watkins *et al*, 1976). This inhibition of renin release was thought to occur via a direct action of calcium on the JG cells, not via the renal nerves or the baroreceptors. Further experiments have increased the evidence in favour of calcium having an inhibitory effect on renin release, in contrast to the more typical findings in exocrine and endocrine cells, where the calcium concentration is usually positively correlated with the secretion rate. In 1977 Baumbach & Leyssac looked at isolated preparations of rat glomeruli. They found that decreasing the extracellular concentration of iCa^{2+} increasd renin secretion, while increasing the intracellular concentration of iCa^{2+} using a calcium ionophore which facilitated calcium influx into the cells inhibited renin release. Others found that chelating extracellular iCa^{2+} with EDTA which subsequently produced a fall in intracellular iCa^{2+} increased renin release (Park & Malvin, 1978). The mechanism(s) by which raised intracellular calcium concentrations inhibit renin release remains hypothetical. One possibility is that calcium may activate chloride channels allowing chloride to flow out of cells followed by potassium, thus the osmotic pressure of the cell falls and water is lost by the JG cell and its renin-containing organelles which therefore are less able to secrete renin. Another hypothesis is that the secretory granules and the cell membrane are separated by a network of myofilaments which contract as the intracellular concentration of calcium is raised, this decreases contact between them and so secretion cannot occur.

This negative association between serum iCa^{2+} and PRC was not apparent in the IDDM women in this study probably because there are other, quantitatively more important, factors acting to suppress renin secretion in IDDM. The most important of these is likely to be that prostacyclin production is impaired in IDDM pregnancy compared with normal pregnancy (Kuhn *et al,* 1990) and prostacyclin is known to be an important stimulator of renin release (section 1.3C, p38). Other factors probably also play a part, for example chronic glycosylation inactivates the prorenin-processing enzyme cathepsin B (Coradello *et al*, 1981) resulting in higher levels of prorenin and low PRC. Another possibility is that the neuroendocrine processes involved in renin production and secretion are affected by IDDM. In 1979, Tuck *et al* demonstrated decreased sympathetic activity and impaired β-receptor mediated responsiveness of

renin release in IDDM patients with peripheral neuropathy. None of the IDDM women in this study had obvious peripheral neuropathy, although nerve conduction studies were not done to look for the condition. However, as mentioned above, the IDDM women as a group failed to show a mid-trimester decrease in DBP and it was postulated that this might be a result of some degree of autonomic neuropathy. It is therefore not unreasonable to postulate that their renin release be impaired by sympathetic nerve dysfunction.

This thesis also found no correlation between serum iCa^{2+} and PRC in the normal pregnant women where basal levels of PRC were two- to three-fold higher than non-pregnant values. The main stimulus to renin release in pregnancy is thought to be the progesterone-driven increased renal excretion of sodium which would rapidly result in sodium depletion if it were not countered by increased renin, and hence aldosterone, production. During normal pregnancy progesterone rises about seven fold from levels found during the luteal phase of the menstrual cycle (Aspillaga, Whittaker, Taylor & Lind, 1983). In IDDM pregnancy levels are even higher (Stewart, Whittaker, Persson, Hanson & Lind, 1989). In the face of such a strong stimulus it is not surprising that the correlation between serum iCa^{2+} and PRC was lost.

Compared with calcium less work has been done on Mg and renin secretion. In isolated, perfused rat kidneys (Fray, 1977; Ettienne & Fray, 1979) and in dog kidneys (Wilcox, 1978) increases in the extracellular concentration of Mg do appear to stimulate renin release. Infusion of Mg to increase basal levels by approximately 50% has also been shown to increase PRA in man (Ichihara, Suzuki & Saruta, 1993). This agrees with this study's findings for the normal non-pregnant women but in the pregnant and IDDM women the situation was confused probably reflecting the fact that in these conditions Mg is not a physiological stimulus for renin release.

This study was unable to find any correlation outside pregnancy between iPTH and PRC in either the normal or IDDM subjects. In contrast to this, in 1992, Grant *et al* provided evidence for a direct stimulatory effect of 1,34-PTH on PRA in non-pregnant human volunteers. Although PRC and PRA are not identical, in the absence of factors known to influence PRA such as treatment with ACE inhibitors, one would expect them to respond to stimuli in similar ways. However it possible that iPTH has

a different effect to 1,34-PTH on renin secretion. To the author's knowledge the influence of iPTH on PRC has not been studied before during pregnancy. This thesis found no relationship between the two variables in normal pregancy

PRS results

The results presented earlier confirm that PRS is unchanged in IDDM women compared to normal women outside of pregnancy. At 21 weeks of pregnancy the IDDM women studied showed pregnancy-induced rises in PRS similar to the normal women (fig. 3.53). Broughton Pipkin *et al* (1982) also found that PRS rose in IDDM pregnancy compared to the normal (non-IDDM), non-pregnant range but they found lower values of PRS in their pregnant IDDM women. It is thought that the rise in PRS, which occurs in pregnancy, is oestrogen-driven, paralleling rises found in women taking oestrogen containing oral contraceptives (Derkx *et al,* 1986). In 1989, Stewart *et al* studying two groups, one English and one Swedish, of pregnant IDDM women, reported that serum oestradiol concentrations were higher at 28 weeks in one group (n=25) of IDDM women compared to normal controls although not in their other group (n=15); by 36 weeks both groups of IDDM had raised oestradiol compared to normal. In this study PRS continued to increase significantly ($p<0.05$) between the second and third trimesters in the normal women, again confirming work by Baker *et al,* 1990; however there was no significant trimester difference in the IDDM women. This suggests that an additional factor may be opposing the stimulatory effect of oestrogen in late IDDM pregnancy so that the rise in PRS is reduced.

What this inhibitory factor might be remains unresolved. This thesis has shown reduced iPTH in pregnant IDDM women and iPTH is known to increase renin secretion as discussed in the introductory section (section 1.3C, p37). However iPTH does not appear to affect PRS (Smith *et al,* 1979) outside pregnancy. The effect of iPTH on pregnant PRS has not been studied before; the results in this study suggest a negative relationship between concentrations of iPTH and PRS in pregnancy. HCG probably increases PRS (Bramfield *et al,* 1990) but again this appears to be raised rather than lowered in IDDM compared to normal pregnancy (Stewart *et al,* 1989). Plasma glucose concentrations were higher in the IDDM women compared to the

normal women but, although this study found a significant negative correlation between plasma glucose and PRS in non-pregnant IDDM women, there was no such association during pregnancy in either normal or IDDM women.

Previously published work does not suggest any role for glucose in the control of PRS so the negative correlation mentioned above was surprising. This study also found an unexpected positive correlation ($p=0.002$) between plasma glucose and PRS in the normal non-pregnant women (fig. 3.59). Closer inspection of the data reveal that there are only five data points for values of PRS of greater than 2.75mcgAngI/ml in the non-pregnant normal women and in fact all of these refer to one woman. If this woman's results are excluded then there is no correlation between PRS and plasma glucose. However, there are no *a priori* reasons for excluding this woman, a normal, normotensive multigravida who delivered a healthy child after an uncomplicated pregnancy. Similarly, there is no reason to suspect laboratory error as these samples were analysed along with approximately 500 others and only the five belonging to this woman were abnormally high. This illustrates the difficulty of studies which involve relatively few subjects as one outlier has a disproportionate effect on results. The negative correlation in the non-pregnant IDDM women cannot be explained away by looking in a similar way at the results of a single woman but again it should be remembered that numbers in the non-pregnant group were less than 20 ($n=13$) because of the difficulty in persuading women with a young baby to return to the laboratory and caution is needed in interpreting apparently significant results.

During normal pregnancy this study showed a significant ($p<0.05$ at 21 weeks and $p<0.01$ at 31 weeks) negative correlation between iPTH and PRS. This was unexpected as when analysed separately the data showed an increase in PRS but no change in iPTH. It is difficult to explain this apparent association physiologically and it may just be a statistical error. Correlations were performed on many variables during this study and, even at a significance level of $p<0.01$, 1% of these "significant" results will have occurred by chance.

When parity was considered as a separate issue in this thesis is was interesting to find that at 21 weeks' gestation the primigravid IDDM women did not have significantly lower PRC compared to the primigravid normal women but that PRC was reduced in

the multiparous IDDM women compared to the normal multiparae. Multiparous preganancy is associated with a greater increase in plasma volume than a first pregnancy (McFadyen, 1989). It is likely that IDDM women do not respond to pregnancy with such a large rise in plasma volume as normal women and this difference may be more apparent in first diabetic pregnancies which could explain why there is less suppression of PRC in the primigravid IDDM subjects. Why there should be a difference between 21 and 31 weeks gestation is not clear. It should also be remembered that as there were only 5 IDDM multiparous women included in the analysis (the sixth was one of the subgroup who developed PE) these parity differences should be interpreted with caution.

PRC and PRS results in the women who developed PE

The PRC values in the IDDM women who developed PE were extremely varied. D3 had undetectable PRC outside pregnancy and in the third trimester, three weeks before she was admitted with PE. In the second trimester although PRC rose from zero it remained low. This IDDM woman had suffered from IDDM for 33 years, the longest disease duration amongst the volunteers, and she had also required laser treatment for diabetic retinopathy. Therefore, out of the 20 IDDM women studied, she was probably the worst case and the low renin levels found in her plasma are in keeping with low PRC reported by others in subjects with complicated IDDM. Notably her renal function, as assessed by microalbumin:creatinine ratio was not abnormal during the pregnancy although it was abnormal when she returned afterwards (at 14mg/mmol). As mentioned in the Results section she should probably be regarded as a case of PE superimposed on IDDM renal disease.

D5 raises extremely interesting possibilities; at 22 weeks she had low levels of PRC and normal levels of PRS. When next studied at 31 weeks, just one week before her admission with PE, her levels of PRC were three- to four-fold higher than those of the non-pre-eclamptic IDDM subjects and her levels of PRS were approximately double theirs. It is possible that these very high levels of PRC were responsible for her rapid rise in BP. D5 was unwilling to give blood samples after her pregnancy which was unfortunate as it would have been interesting to see if her PRC returned to normal.

D10's results were inconclusive; in the second trimester and outside pregnancy she had relatively normal values of PRC and PRS but in the early third trimester PRS fell and PRC became undetectable. She did not develop PE until 37 weeks gestation and as this study's protocol did not include PRC estimation in the latter part of the third trimester it is not possible to draw any conclusions from this.

4.7 Summary of findings

This thesis has confirmed the findings of others by showing a mid-trimester fall in diastolic blood pressure in normal pregnancy. Following work from South America on women with calcium deficient diets there was great hope in the 1980s and early 1990s that simple dietary manipulation with calcium supplementation might reduce the incidence of pre-eclampsia and pregnancy-induced hypertension. This now seems not to be the case, at least in women with already adequate calcium intakes, and this thesis supports the latter opinion. The dietary results presented here show that both normal and insulin-dependent diabetic women in the South West of England have dietary calcium intakes in line with the 1991 recommendations of the Committee on Medical Aspects of Food Policy.

This thesis was designed to compare insulin-dependent diabetic pregnancy with normal pregnancy. Inevitably therefore the results presented for normal pregnancy do little but confirm previous work. For example serum concentrations of electrolytes and creatinine are known to fall in pregnancy whilst creatinine clearance and urinary calcium excretion are known to rise. It is also well-established that serum urate concentrations rise with increasing gestation. This thesis also confirms that serum ionized calcium concentrations are stable during normal pregnancy. Recent work on serum concentrations of *intact* parathyroid hormone in normal pregnancy has contradicted the long-held view of pregnancy as a state of physiological hyperparathyroidism. The results of this thesis confirm that intact parathyroid hormone concentrations do not rise in normal pregnancy. This thesis is in agreement with previously published work showing that both plasma renin concentration and plasma renin substrate rise in normal pregnancy.

Similarly, much work has been done on calcium and magnesium homeostasis in diabetic individuals outside pregnancy. Thus the findings in this thesis, outside pregnancy, of low serum concentrations of magnesium and an increase in the fractional excretion of magnesium in diabetic subjects compared to controls are not new. The consensus of opinion is that this urinary loss of magnesium is reversed with improved diabetic control and in this thesis this was certainly the case with the improved diabetic control achieved by the women during pregnancy. Existing data on

serum concentrations of ionized calcium in diabetic subjects outside pregnancy are conflicting; this thesis shows that they are identical to values in non-diabetic subjects.

When normal and insulin-dependent diabetic women were compared during pregnancy this thesis confirmed the previously noted trend for an increased incidence of pre-eclampsia in diabetic pregnancy. It also showed that pregnant diabetic women do not have the normal mid-trimester fall in blood pressure; in this respect the pregnant diabetic women in this thesis are similar to non-diabetic pregnant women who develop pre-eclampsia. A further new finding was that calcium supplementation has no effect on blood pressure in diabetic pregnancy.

This thesis has extended existing knowledge in the field of calcium, magnesium and phosphate homeostasis in insulin-dependent diabetic pregnancy. It has shown that in well-controlled pregnant diabetic women serum concentrations of ionized calcium, phosphate and magnesium are the same as in normal pregnant women and that they respond similarly to an oral calcium load. It has also shown no difference in the fractional excretion rates of either calcium or magnesium between normal and diabetic pregnancy. The fractional excretion of phosphate was increased in diabetic pregnancy.

The response of serum intact parathyroid hormone to oral calcium loading has not been studied before in insulin-dependent diabetic pregnancy. This thesis showed significantly lower fasting levels of intact parathyroid hormone at 21 weeks in diabetic, compared with normal, pregnancy and there was a similar trend at 31 weeks. Following oral calcium intact parathyroid concentrations were suppressed within an hour in both the normal and diabetic subjects and there was a trend towards greater suppression in the diabetic group. Vitamin D has also not been studied adequately in insulin-dependent pregnancy. This thesis only measured 1,25-dihydroxy-cholecalciferol in a very small number of women but did show a trend towards lower levels in the diabetic women which warrants further investigation (section 4.8, p208).

Other positive new findings in this thesis were that plasma renin concentration is not increased in diabetic pregnancy as it is in normal pregnancy, and that plasma renin substrate rises in diabetic pregnancy as it does in normal pregnancy. The rise in

plasma renin concentration which occurs in normal pregnancy is thought to be a response to the progesterone-driven natriuresis of pregnancy. This thesis showed no reduction in sodium excretion in diabetic pregnancy which raises the question that an additional factor may be acting to suppress plasma renin in pregnant diabetic subjects. The similar rise in plasma renin substrate in normal and diabetic pregnant women was not unexpected as there was no *a priori* reason for thinking that plasma renin substrate levels are affected by insulin-dependent diabetes.

This thesis also found a trend towards reduced plasma renin concentration in multigravid diabetic women compared to diabetic primigravidae which has not been reported before. This raises interesting questions but may in fact be merely an error resulting from the small numbers of multigravid diabetic women in the study.

Finally the findings of extremely high levels of both plasma renin concentration and plasma renin substrate in one of the diabetic women just one week before the onset of clinically apparent pre-eclampsia, whereas her levels earlier in the pregnancy were indistinguishable form those of the other pregnant diabetic women, raises interesting possibilities about the role of the renin-angiotensin system in the aetiology of pre-eclampsia.

4.8 Suggestions for further research

Although this thesis has answered many of the questions posed initially regarding both calcium homeostasis and the RAS in IDDM pregnancy it has also raised more queries and highlighted areas of deficiency in the current understanding of the pathophysiology of IDDM pregnancy. Before discussing some of these areas in greater detail a few general points are worth considering.

The greater value of prospective, longitudinal research compared to both retrospective and cross-sectional studies is not disputed. Whilst it is relatively easy to study women longitudinally during pregnancy, a time when frequent visits to hospital are well-tolerated, it is much harder to continue a study into the puerperium. It is also difficult to recruit large numbers of pregnant women with IDDM over a short time in a small area. This study managed to follow 20 normal and 20 IDDM women during pregnancy but less were willing to return after delivery, especially because of the prolonged wait for breast feeding to be discontinued. Numbers recruited would have been higher had the study not involved the randomization to either calcium or placebo as some women approached declined to accept this. As this thesis has shown no effect of calcium supplementation on the women studied, who already had a diet adequate in calcium, any future study could leave out the calcium supplement part of this work and recruitment would therefore be easier. Recruitment could also have been improved by extending the study to neighbouring hospitals, thus achieving higher numbers without jeopardizing the prospective, longitudinal aspect of the study.

Women with pre-existing hypertension or renal disease were not recruited for this thesis. If a larger study were being undertaken it would be interesting to deliberately recruit such a sub-group for comparison with the uncomplicated IDDM women.

Analysis of the results in this thesis, and comparison of them with the results of others, was potentially complicated by the fact that the women recruited were of mixed parity, although in many cases this did not significantly alter findings. In general any study on pregnancy should involve either only primigravid patients or large and equal numbers of primigravidae and multigravidae to facilitate statistical analysis.

This study found that serum concentrations of iPTH are low in IDDM pregnancy and this immediately raises questions about differences in other calciotrophic hormones. The limited number of 1,25-DHCC assays which were performed suggest that this too is reduced in IDDM pregnancy and it would be interesting to measure this in a larger number of women. Outside pregnancy 1,25-DHCC is produced in the kidneys; during pregnancy the placenta acts as an additional source of the vitamin as well as producing 24,25-DHCC (Weisman *et al,* 1979; Whitsett *et al,* 1981). It is likely that the IDDM placenta differs from the normal placenta and may therefore not produce so much 1,25-DHCC. It would be extremely interesting to compare *in vitro* production of the various forms of vitamin D in normal and IDDM placental preparations and also to measure circulating concentrations of the same substances *in vivo.*

This thesis has shown that PRC is reduced in IDDM pregnancy as it is in non-pregnant IDDM individuals (Day *et al,* 1975; Luetscher *et al,* 1985). However in normal pregnancy PRC is raised (Skinner *et al,* 1972; Oats *et al,* 1981; Baker *et al,* 1990) and in order to compensate for this sensitivity to infused Ang II is reduced (Talledo *et al,* 1967). Subsequently *increased* sensitivity to infused Ang II has been shown to be predictive of the development of PE (Morris *et al,* 1978; Oney & Kaulhausen, 1982; Nakamura *et al,* 1986; Dekker *et al,* 1990). Having demonstrated alterations in components of the RAS in IDDM pregnancy further work should now be done on the sensitivity of pregnant IDDM women to infused Ang II.

The blood pressure results presented in this thesis showed that the IDDM women did not have a "mid-trimester fall" in diastolic blood pressure and it was suggested that this was because of autonomic dysfunction leading to a reduced fall in peripheral resistance compared to normal pregnant women. Recent use of 24 hour ambulatory blood pressure monitoring has shown that non-pregnant IDDM subjects, who are apparently normotensive on conventional measurement and who have no evidence of renal impairment, are less likely to have an overnight fall in blood pressure and that this correlates with their degree of autonomic nerve impairment (Spallone *et al,* 1994). Pregnant women with PE similarly have no overnight fall in blood pressure (Halligan *et al,* 1997). A future study performing prospective 24 hour ambulatory blood pressure monitoring on a group of pregnant IDDM women and simultaneously looking at their autonomic nervous function might well produce interesting results.

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APPENDIX I

Patient information sheet and consent form (see over) signed by volunteers before participating in study.

PATIENT INFORMATION SHEET FOR RESEARCH STUDY ON CALCIUM IN NORMAL AND DIABETIC PREGNANCY

Calcium is an important part of the human body. It forms the major part of bone and is also found circulating in the blood where it is involved in the process of muscle contraction. It is also important for the contraction and dilation of blood vessels and so has an effect on blood pressure.

It is very important that mothers take enough calcium in their diet during a pregnancy so that the baby is provided with all that it needs especially for the development of its skeleton.

There seem to be differences in the ways in which the bodies of pregnant diabetic women deal with calcium compared to non-diabetic women. For example, babies born to mothers with diabetes may have low levels of calcium in their blood. Diabetic women are also more likely to have problems with high blood pressure during a pregnancy and this may be related to the way in which their body handles calcium.

This research study is planning to look at levels of calcium and related substances in the blood and urine of pregnant diabetic women and also in some non-diabetic pregnant women so that we can compare results and see why the diabetic group is more likely to have high blood pressure.

If you agree to take part in this research you will either be given calcium supplements or a placebo (ie. a look-alike tablet which does not contain any calcium) from 20 weeks of pregnancy. Neither of these tablets will have any harmful effects on your baby. Your blood pressure will be measured every two weeks in the normal way.

In order to study more closely the effects of the calcium tablets on substances in your blood and urine you will be asked to participate in two short studies during the pregnancy and in one after your baby is born. This will involve collecting urine at home for 24 hours. The next day you come up to hospital for a morning during which some blood and urine samples will be taken. You will be given breakfast and a drink containing calcium. After lunch, which will be provided, you will be free to go home.

CONSENT FORM

RESEARCH STUDY ON CALCIUM IN NORMAL AND DIABETIC PREGNANCY

Have you read the patient information sheet? YES/NO

Have you had the opportunity to ask questions and discuss the study? YES/NO

Have you received satisfactory answers to all your questions? YES/NO

Have you received enough information about this study? YES/NO

Do you understand that you are free to withdraw from this study
- at any time
- without having to give a reason
- and without affecting your future medical care? YES/NO

Do you agree to take part in this study? YES/NO

Signed:_____ Date:_____

Name (in block letters):_____

APPENDIX II

Questionnaire used to assess dietary calcium intake.

1) What sort of milk do you use?
   eg. whole, skimmed, semi-skimmed, fortified eg. Calcia, evaporated
2) How much milk do you use daily including tea and coffee?
3) Which bread do you use?
   eg. white, brown, wholemeal, high fibre white eg. Mighty White
4) Do you use small or large loaves? or rolls?
   Do you use thin, medium or thick sliced?
   How many slices per day?
5) Do you drink bottled (what brand) or tap water?
   How much?
6) How many drinks do you have per day made with water?
7) Do you use a water filterer or softener?
8) Do you eat yoghurt (Greek / natural / fruit) or fromage frais?
   How much per week?
9) Which hard cheeses do you eat?
   How much per week? (a food portion model was used)
10) Which soft cheeses do you eat?
    How much per week? (again a model was used)

Using food portion models where appropriate the amount of the following foods eaten per day / week / fortnight / month was then recorded:

Ice cream
Eggs
Fish - Salmon
Sardines
Shellfish
White
Oily / tuna

Nuts

Biscuits

Fresh orange juice

Dried fruit

Dark green vegetables

Milk pudding / custard

Pastry (white or wholemeal)

Cakes (specify)

Pizza

Beans - baked

kidney

other

Soups (cream variety or home-made with milk)

Milky drinks eg. milkshakes

White sauce

The subjects were also asked if they took calcium or mulitvitamin supplements, and antacids or indigestion tablets.

APPENDIX III

Details of suppliers:

Johnson & Johnson Clinical Diagnostics Inc - 100 Indigo Creek Dr
Rochester NY 14650
USA

Bio Rad Laboratories - ECS
3726 East Miraloma Av
Anaheim CA 92806
USA

AVL analyser - AVL Medical Instruments UK Ltd
Whitebridge Lane
Stone
Staffs ST15 8LQ

Ciba Corning Diagnostics - Rochester Rd
Halstead
Essex CO9 2DX

DAX 48 - Bayer Diagnostics
Evans House
Hamilton Close
Basingstoke
Hants RG21 2YE

Hepsal - CP Pharmaceuticals Ltd
Ash Rd North
Wrexham Industrial Estate, Wrexham
Clwyd LL13 9UF

Cacit - Norwich Eaton
PO box 1YD
Newcastle upon Tyne NE99 1YD

Calcichew - Shire Pharmaceuticals Ltd
1 Viscount Ct
South Way
Andover SP10 5NW